Késsia Hapuque Santos de Andrade

Licenciatura em Bioquímica na Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa

Cyclopentenones: synthesis and biological evaluation in human cancer cells

Dissertação para obtenção do Grau de Mestre em Química Bioorgânica

Orientador: Prof. Doutor Carlos Alberto Mateus Afonso, Professor Catedrático, Faculdade de Farmácia da Universidade de Lisboa
Co-orientador: Rafael Filipe Teixeira Arbuéz Gomes, Investigador, Faculdade de Farmácia da Universidade de Lisboa

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FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

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Resumo

Ciclopentenonas (CPs) são cetonas cíclicas cuja estrutura consiste num grupo carbonilo α,β-insaturado ligado a um anel. Muitos compostos que contêm o anel de ciclopentenona são biologicamente ativos, como por exemplo as prostaglandinas que apresentam propriedades antivirais, antiinflamatórias, antifúngicas, apoptóticas e antitumorais.

Foi demonstrado que a maioria dessas propriedades são devido ao caracter aceitador de Michael do grupo carbonilo α,β-insaturado que pode reagir com diversos nucleófilos. Um exemplo é a reação com Glutationa, que atua em mecanismos de desintoxicação, contribuindo positivamente para combater a resistência à medicamentos antitumorais. No entanto essa reatividade desses aceitadores de Michael os torna promíscuos à reações indesejadas com macromoléculas perigosas para as células.

Tendo por base essa promiscuidade, o nosso objetivo foi de sintetizar uma pequena biblioteca de compostos com pobre caracter aceitador de Michael e que apresentam citotoxicidade em células tumorais do colon retal, da mama e do pulmão. Sendo assim, preparamos diferentes CPs substituídas nas posições 2 e 4, baseando as nossas reações em métodos já descritos, inclusive pelo nosso grupo.

Dessas CPs, as 2-morfolino CPs e as 2-hidroxi CPs não formaram aductos com Glutationa por adição de Michael provando ser pobres aceitadoras de Michael e mostraram considerável atividade citotóxica contra as células tumorais testadas. Já a *Trans*-4,5-morfolino ciclopentenona formou aducto com Glutationa provando ser um ótimo aceitador de Michael ao mesmo tempo que não apresentou nenhuma actividade citotóxica.

Apesar do trabalho ainda estar em andamento podemos inferir que as CPs podem actuar por um mecanismo de ação diferente dos conhecidos por alquilação ao grupo carbonilo α,β-insaturado, e isso e um grande avanço no âmbito da química medicinal para a descoberta de novos fármacos com melhor desempenho contra o cancro e menores efeitos secundários.

Palavras chave: Ciclopentenona, glutationa, cancro, citotoxicidade, aceitador de Michael

Abstract

Cyclopentenones (CPs) are cyclic ketones whose structure consists of an α, β-unsaturated carbonyl group attached to a membered ring. Many compounds which contain the cyclopentenone ring are biologically active, for example prostaglandins which have antiviral, anti-inflammatory, antifungal, apoptotic and antitumor properties

It has been shown that most of these properties are due to the Michael acceptor character of the α, β-unsaturated carbonyl, group which can react with various nucleophiles. An example is the reaction with Glutathione, which acts on detoxification mechanisms, contributing positively to combat resistance to antitumor drugs. However, this reactivity of these Michael acceptors makes them promiscuous to unwanted reactions with macromolecules critical to cells.

Based on this promiscuity, our goal was to synthesize a small library of compounds with poor Michael acceptor character and which have cytotoxicity in tumour cells of rectal colon, breast and lung. Thus, we prepared different substituted CPs in positions 2 and 4, basing our reactions on methods already described, including by our group.

From these synthesized CPs, 2-hydroxy-4-substituted CPs (with lower Michael acceptor character) showed considerable cytotoxic activity, while the others, showed no sufficiently cytotoxicity. The CP with a very good Michael acceptor character formed adducts with Glutathione in stability assays, while the 2-morpholine-4-substituted CPs (with lower Michael acceptor character) showed no formation of adducts with Glutathione.

Although the work is still in progress we can infer that CPs can act by a mechanism of action different from those known by alkylation to the α, β-unsaturated carbonyl group, and this is a great advance in the field of medicinal chemistry for the discovery of new drugs with better cancer performance and fewer side effects.

Keywords: Cyclopentenone, glutathione, cancer, cytotoxicity, Michael acceptor

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List of Abbreviations

ACN – Acetonitrile

BHK-2 – baby Hamster Kidney Cells

Boc – tert-butyloxycarbonyl protecting group

CAN – Ceric ammonium nitrate

CP – Cyclopentenone

CPPGs – Cyclopentenone Prostaglandines

COMC – 2-Crotonyloxymethyl-2-cyclohexenone

COTC – -(*4R, 5R, 6R*)-4,5,6-trihydroxy-2-cyclohexenone

DBU – 1,8-Diazabicyclo[5.4.0]undec-7-ene

DMAP – 4-Dimetilaminopiridina

DMF – Dimethylformamide

DMSO – Dimethyl Sulfoxide

DNA – Deoxyribonucleic acid

EtOAc – Ethyl Acetate

E1cB – **E**limination **U**nimolecular **c**onjugate **B**ase

Glo-I – glyoxalase I

GSH – Glutathione

GST – Glutathione S-transferase

HPLC – High Performance Liquid Chromatography

IGF-1 – Insuline Like Grownt Factor 1

IKK-β – Nuclear Factor Kappa B

IUPAC – International Union of Pure and Applied Chemistry

JNK – c-Jun N-terminal kinases

KO^tBu – Potassium Tert-butoxide

LA – Lewis Acid

L1210 – a mouse lymphocytic leukemia cell line

MDR – Multidrug resistance

NBS – N-Bromosuccinimide

NF-κB – factor nuclear kappa B

NMR – Nuclear Magnetic Resonance

PDC – Pyridinium Dichromate

PGs – Prostaglandins

PTSA monohydrate – p-Toluenesulfonic Acid Monohydrate

RPMI medium – Roswel Park Memorial Institute medium

TEA – Triethylamine

TFA – Trifluoroacetic acid

TLC – Thin Layer Chromatography

TMS – Tetramethylsilane

Traf-2 – Tumor Necrosis factor receptor associated factor

SAR – Structure Activities Relationships

SES – 2-(Trimethylsilyl)ethanesulfonyl

SSs – Stenhouse salts

1

Introduction

1.1. Medicinal Chemistry

The medicinal chemistry is an interdisciplinary science concerned on “the discovery, the development, the identification and the interpretation of the mode of action of biologically active compounds at the molecular level” (IUPAC). In general, encompasses a combination of chemistry (synthetic organic chemistry and computational chemistry) and biological sciences (molecular biology, biochemistry, enzymology, pharmacology, veterinary and human medicine, and toxicology).[1]

The synthetic organic chemistry is focused on the design and synthesis of new therapeutic agents (using natural products or commercially available molecules as starting material) through novel chemical reactions and on the optimization of these reactions such as: 1) reaction selectivity; 2) energy required; 3) yield; 4) reaction times; 5) costs; 6) safer conditions; 7) minimize the environmental impact.

The computational chemistry uses theoretical chemistry and computer simulation methods to solve chemical problems and understand Structure Activities Relationships (SAR).

Biological Sciences study the biological response to the novel molecules and their mechanism, using a wide range of methodologies according to the purpose.[2]

From ancient times, human beings strive to discover the cure of diseases that affect them. Before the twentieth century, they used to treat diseases by rudimentary techniques such the use of medicinal herbs and potions, but the medicinal chemistry born in the mid-nineteenth century with the first serious efforts to isolate and purify the pure chemicals responsible for the medicinal properties (active principles) of remedies. Since then, it is possible to obtain many naturally occurring drugs and determine their structures (Figure 1.1.). [2,3] Nowadays, with the advances in chemical and biological

sciences it's possible to understand better body functions at cellular and molecular level, and discover, design and develop drugs that can combat many diseases.

One important group of diseases that have been extensively studied by the scientific community is Cancer.

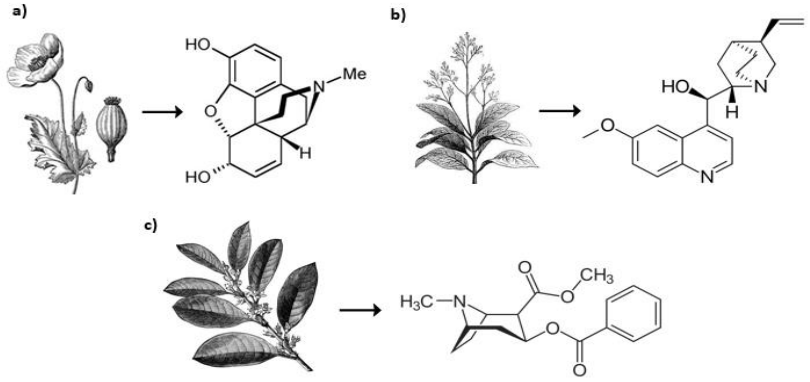


Figure 1.1. Examples of natural occurring bioactive molecules: (a) *morphine from opium; b) quinine from the bark of the cinchon; c) cocaine from coca leaves*

1.2. Cancer, apoptosis and Multidrug resistance

Cancer is a generic term for a group of diseases characterized by the uncontrolled division of abnormal cells, which cannot suffer apoptosis and may invade adjacent parts of the body and spread to other organs.

Environmental influences, dietary influences and genetic polymorphisms are the factors that most contribute to the development of cancer. The latter involves the alteration or deletion of genes such as oncogenes, tumour suppressor genes, DNA repair genes and genes encoding phase I and phase II enzymes.[4,6]

The apoptosis is an essential physiological process of regulated destruction of cells, being important for cell migration and cell division, for the maintenance of tissue homeostasis.[7] Morphologically, the process involves rapid cell condensation followed by formation of membrane-enclosed apoptotic bodies containing well-preserved organelles, which are phagocytosed and digested by nearby cells **(**Figure 1.2.).[8]

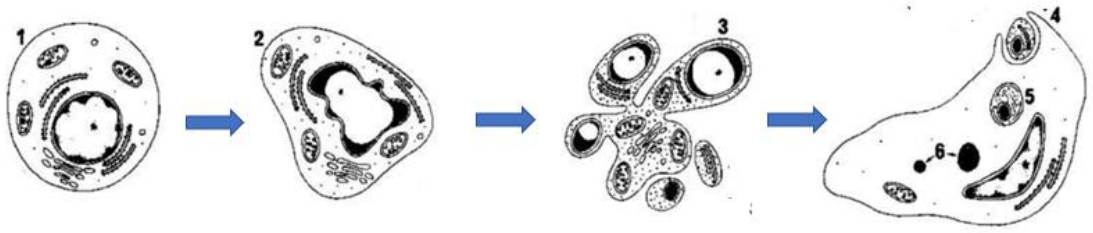


Figure 1. 2. Diagram illustrating sequence of ultrastructural changes in apoptosis. (1) Normal cell. (2) Early apoptosis characterized by compaction of chromatin, condensation of cytoplasm, and convolution of nuclear and cell outlines. (3) The nucleus fragments and protuberances formed, separate to produce apoptotic bodies. (4, 5, 6) These bodies are phagocytosed by nearby cells and degraded within lysosomes. Adapted from Kerr, J.F.R. et al, Cancer, 1994, 73 (8).[7]

The activity of many genes influences a cell's probability of activating its self-destruction programme and, when the decision to undergo apoptosis is made, a proteolytic cascade is triggered in the suicidal cell. This cascade involves the activation of cysteine proteases known as caspases that cleaves different substrates, activating and inactivating proteins. One example is the activation of nucleases that degrade DNA generating fragments which are a marker for apoptotic cell death. There have been described three main mechanisms for the activation of caspases: 1) proteolytic cleavage by an upstream caspase, 2) induced proximity and 3) holoenzyme formation. These mechanisms actuate harmoniously into the proteolytic cascades (Figure 1.3.). [7,9,10]

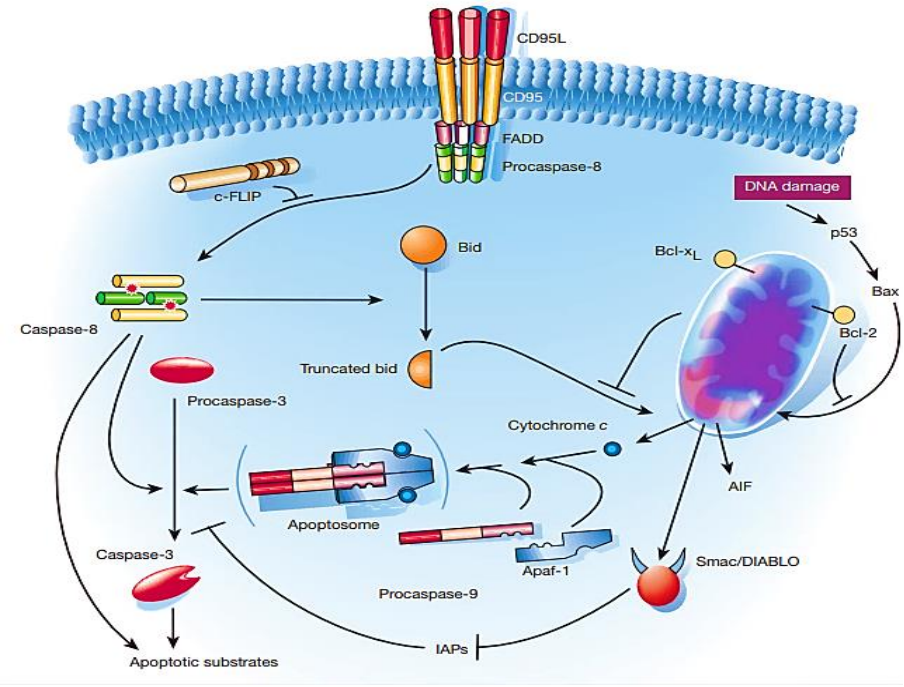


Figure 1.3. Example of two major apoptotic pathways in mammalian cells. Both culminate at the cleavage of apoptotic substrates. The left is death-receptor pathway where the caspase-8 are activated by induced proximity and the right is the mitochondrial pathway used extensively in response to extracellular cues and internal insults such as DNA damage where cascase-3 are activated by different ways. Reproduced from Budihardjo, I. et al, Annu. Rev. Cell Dev. Biol., 1999, 15.[9]

Targeting the cell apoptosis and consequently the cancer treatment considerable number of pharmaceutical drugs have been developed. Unfortunately, a big problem involves the cancer chemotherapy: the multidrug resistance (MDR).[11]

MDR is the ability of cancer cells to acquire resistance to various drugs.[11] Several mechanisms have been encountered for the acquisition of resistance by tumor cells. One of these is the induction and activation of efflux transporter proteins.[12] Others are: the mutations in topoisomerase II,[13] the enhanced repair of DNA,[14] the changes in genes that are critical for proliferation or apoptosis,[15] and overexpressing enzymes that may increase detoxification and circumvent the cytotoxic action of antitumor drugs.[16]

1.3. The Worldwide Problem of cancer

Every year, around the world, millions of people are diagnosed with cancer, and more than half of the patients die from it. According to the estimates for 2011 from World Health Organization (WHO), cancer is considered the second most common cause of death after cardiovascular disease being responsible for 8.8 million deaths in 2015.[5] The incidence of cancer is increasing, with more than 20 million new cases of cancer expected in 2025. This increase of cancer disease is related to the continuing global demographic and epidemiologic transitions. Thus, with the growth, the population aging (older people are more susceptible to cancer) and the economic development, mainly in developed countries, some habits are risk factors that contribute to increase the incidence of these disease cases. These habits can be a sedentary life-style, stress, bad eating habits and smoking. If these risk factors could be modified between 30% and 50% of cancer deaths could be avoided.[17-21]

GLOBOCAN is a database assembled using an enormous amount of data available from the Descriptive Epidemiology Group of the International Agency for Research on Cancer (IARC), an agency of the World Health Organization in Lyon, France. The fifth version of GLOBOCAN estimates, per sex, the incidence and mortality rates in 2012 for all cancers, in 184 countries and 30 regions worldwide (Figure 1.4.)[17]. The data point to lung cancer (1.82 million), breast cancer (1.67 million) and colon cancer (1.36 million), as the most commonly diagnosed cancers (excluding the non-melanoma skin cancer), and also point to lung cancer (1.6 million deaths), liver cancer (745,000 deaths) and stomach cancer (723,000 deaths) as the most responsible cancers to deaths.[22] The developing countries have greater problems in diagnosing the number of cases and consequently the number of deaths by cancer, but developed countries show higher rates of cancer. This is due to the same risk factors mentioned above: aging of the population and growth. Should be mentioned that the type of cancer varies between geographic regions and the factors could be for the economic development, for the genetic differences and lifestyle differences. [17-21]

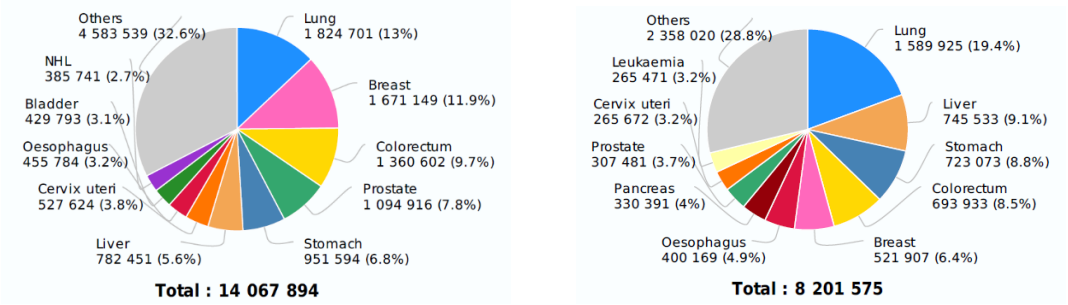


Figure 1.4. – At left are represented the estimated number of incident cancer cases, both sexes, all ages, worldwide (top 10 cancer sites) in 2012, and at right the estimated number of deaths by cancer, both sexes, all ages, worldwide (top 10 cancer sites) in 2012. Reproduced from: GLOBOCAN 2012, Global Cancer Observatory, International Agency for Research on Cancer 2018.[17]

1.4. Cyclopentenones as antitumor agents

Cycloalkenones, such as cyclopentenones and cyclohexenones are an important class of molecules that have been thoroughly studied as a highly versatile building blocks for the synthesis of many natural and synthetic biologically active compounds.

Cyclopentenones (CP) are cyclic ketones whose structure consists of an α, β-unsaturated carbonyl group attached to a ring which may suffer many modifications. (Figure 1.5.). One example of these are the additions. The way that nucleophiles reacts on additions depends on the nature of the α-β-unsaturated carbonyl, the type of nucleophile and the reactions conditions.[23]

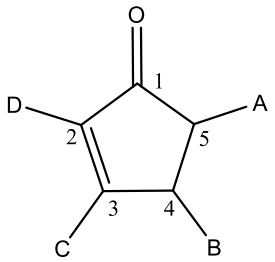


Figure 1.5. – 5-membered ring structure of CP

The CP structure has been shown to be indispensable for the biological activity of some compounds mainly due to the α-β unsaturated carbonyl group. This group is an electrophilic centre susceptible of undergoing addition reactions with nucleophiles such as sulfhydryl groups of reduced glutathione (GSH), free cysteine residues in proteins and other macromolecules such as DNA. [24-28]

1.5. Cyclopentenones Prostaglandins (CPPGs)

Prostaglandins PG(s) are chemical messengers synthetized from fatty acids, mainly having diverse hormone-like effects. They act on a variety of cells as autocrine and paracrine lipid mediators. These different biological activities depend on their structure and consequently on the type of receptor which it binds.[26,29]

The cyclopentenones prostaglandins (CPPGs) are a PGs subgroup which are part the class A and J, coming from dehydration of class E and D respectively (Figure 1.6.).[30,32] The CPPGs may interact with multiple cellular targets including signalling molecules and transcription factors and they show antiviral,[25,33] anti-inflammatory,[24,34] antifungal,[35] apoptotic[36-38] and antitumor properties.[39-41]

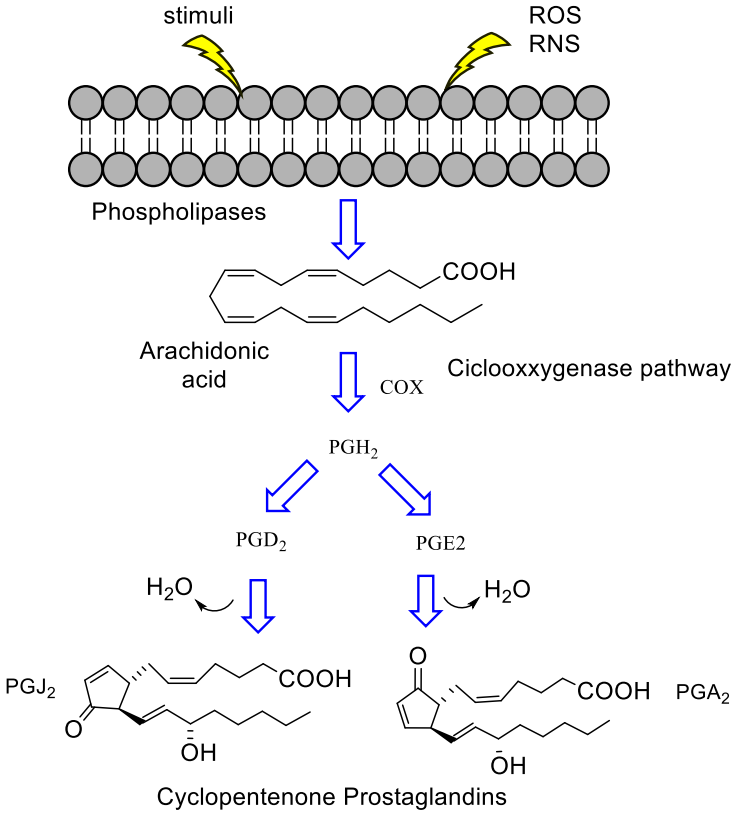


Figure 1.6. – Generation of cyclopentenone prostaglandins in the metabolism context. Adapted from Garzon, B. et al, J Proteomics, 2011, 74.[95]

Studies with CPPGs showed that the CP moiety act as a pharmacophore of anticancer molecules.

The first evidences of these have showed that PGE2 causes inhibition of DNA synthesis in BHK-2 cells[42] but further was found that the growth inhibition is exerted by the dehydration product

PGA2 and the same results was demonstrated into PGJ2 and its precursor PGD2 (with stronger inhibition of PGJ in relation of PGD).[31] Studies with other related compounds with no cyclopentenone ring showed no similar range of biological activities, for example observations by Bui and Strauss on the effect of CPPGs on stress-induced IGF-1 and Waf1 gene expression. Unlike the compounds without the cyclopentene moiety, these CPPGs repressed IGF-1 and induced Waf1 gene expression, demonstrating the requirement for the α-β unsaturated carbonyl group for the regulation of these two genes.[43] This regulation of IGF-1 and p21CIP1 / WAF1 by CPPGs are associated with the cell cycle arrest, inducing growth arrest of human and murine tumor cells in the G1 phase at sub-toxic doses.[44-46]

These results demonstrate directly that CPPGs requires the reactive α-β unsaturated carbonyl group for biological activity and it's possible to state that the majority of its activities are mimicked by 2-cyclopenten-1-one itself.[24,41,48,49] This requirement suggests that the CPPGs might alkylate exposed cysteine residues on key cellular target proteins and this alkylation may result in loss of function of the targets.[49]

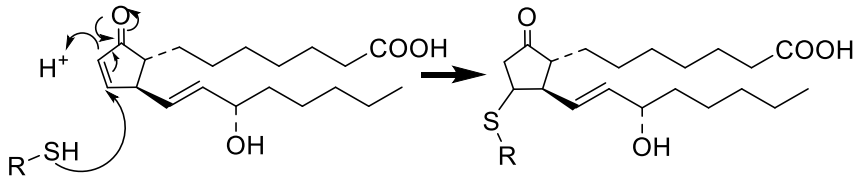
This alkylation with specific cysteine residues in cellular proteins might be confirmed by the elucidation of the molecular mechanism for repression of the NF-κB activity. The NF-κB is a transcription factor involved in inflammation and immunity, activated in response to proinflammatory stimuli, stress or infection. This pathway activation has been associated with stimulation of cancer cell proliferation and prevention of apoptosis.[50,51] CPPGs may inhibit this pathway at several levels. One of these is the direct modification of a critical cysteine residue in IKK-β (this enzyme phosphorylates the NF-κB inhibitory molecule, releasing it from the complex and allowing it to be transported to the nucleus to promote DNA transcription)[34] and the other is the direct interaction with the p50 subunit of NF-κB inhibiting DNA binding.[52,53]

Corroborating these studies, naturally occurring CP marine prostanoids, such as clavulones and derivatives demonstrated a cytotoxicity comparable to the best performing chemotherapeutics.[54] Furthermore, scientists showed that incorporating the CP chemical group in anticancer molecules such as chalcones[55] and jasmonates[56] increases the antiproliferative potential.

All these findings led us to conclude that cyclopentenone prostaglandins serves as a simple model compounds for studies about the biological activity of many Michael acceptors (with the α-β -unsaturated carbonyl group).[26,57,58]

1.6. The reaction with Proteins and Reduced Glutathione (GSH)

The CPPGs may also react with the sulfhydryl group of reduced glutathione (GSH) (Scheme.1.1.).



Scheme 1.1. - Alkylation of PGs by thiol molecules.

Glutathione (GSH) is a tripeptide produced naturally in the body found within almost all cells and being the most abundant non-protein thiol in the cell.[59] This peptide is synthesized from just three amino acids, glutamic acid, cysteine and glycine and has several different and vital physiological roles including antioxidation, maintenance of the redox state, modulation of the immune response and detoxification of xenobiotics. Under normal conditions, the majority of GSH exists in reduced form and a minority in the oxidized form (GSSG).[60] This last is obtained from the oxidation of the reduced by direct interaction with free radicals or acting as a cofactor for antioxidant enzymes**.** GSH may also be found conjugated to protein and non-protein sulfhydryl, via disulphide bond formation.[61,62]

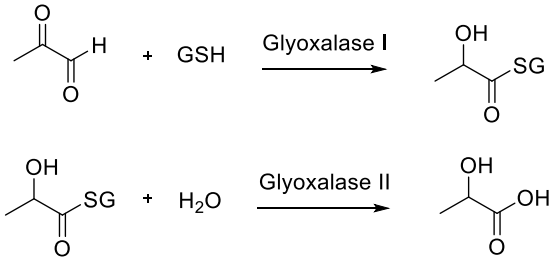
In cancer GSH has both protective and pathogenic roles. It is crucial in the removal and detoxification of carcinogens,[63,64] but in cancer cells may also increase resistance to chemo- and radiotherapy.[61]

Many detoxifications of carcinogens mechanisms include phase I reactions catalyzed by cytochrome P-450, hydrolysis or reduction, and phase II reactions. The GSH conjugation with electrophilic substances such as alkenes, halides and epoxides act as the major phase II reactions in mammalian species. This conjugation is usually catalyzed by phase II isoenzymes known like glutathione S-transferases (GSTs) that in human are divided into seven classes (Alpha, Mu, Pi, Omega, Theta, Zeta and microsomal),[65,66] being that the most highly expressed GST isoenzyme in various human cancerous and precancerous tissues is GSTP1-1.[67]

These enzymes are also associated to a detoxification process of anticancer drugs, such as chlorambucil,[68] melphalan,[69] carmustine,[70] cisplatin,[71] and busulfan,[72] reducing drastically the reactivity of these compounds and favoring their elimination by glutathione S-conjugate export pump (GS-X pump).[61,73-77] The GSTs, specifically GSTP1 also act on the regulation of apoptosis through inhibition of signaling pathway.[78] In the absence of stimuli, GSTP1-1 is bound to enzymes, such as

Traf-2 and JNK, which upon oligomerization of GSTP1-1, are released in order to play their role in inducing apoptosis.[79] Therefore, the overexpression of GSTs and the high levels of glutathione (GSH) in tumors are associated to the development of MDR.[61,73-77] Thus, in order to modulate cancer cell resistance to anticancer drugs the search for molecules capable of inhibiting GS-X pump, GSTs and Glo-I has become one of the primaries aims of contemporary research.

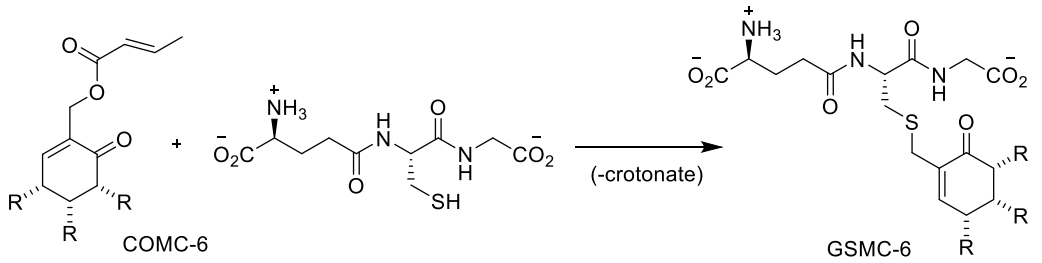
Among other detoxification reactions that glutathione acts, we draw special attention for the detoxification of methylglyoxal (one toxin produced as a product of metabolism) performed by the glyoxalase system that involves the reduction of glutathione to S - D - lactil – glutathione (catalysed by glyoxalase-I) and its hydrolysis to glutathione and D-lactic acid (catalysed by glyoxalase-II).[80,81] Studies have showed that methylglyoxal is toxic to proliferating cells and has been suggested that Glo-I are associated to drug resistance since has been detected in elevated levels in tumour colon tissue (Scheme 1.2.).[82]



Scheme 1.2. - Reaction of methylglyoxal detoxification

It was thought that the inhibition of a Glo-I was responsible for the anticancer properties.[83,84] Thus, in 1975, a potential inhibitor of glyoxalase-I was isolated by Umezawa and co-workers from the culture broth of *Streptomyces griseosporeus* known as 2-crotonyloxymethyl-(*4R, 5R, 6R*)-4,5,6-trihydroxy-2-cyclohexenone (COTC),[85,86] but Umezawa *et al.* noted that the inhibition of the enzyme was observed only in the presence of GSH, in other words, the COTC had no significant effect by itself.[85,87,88]

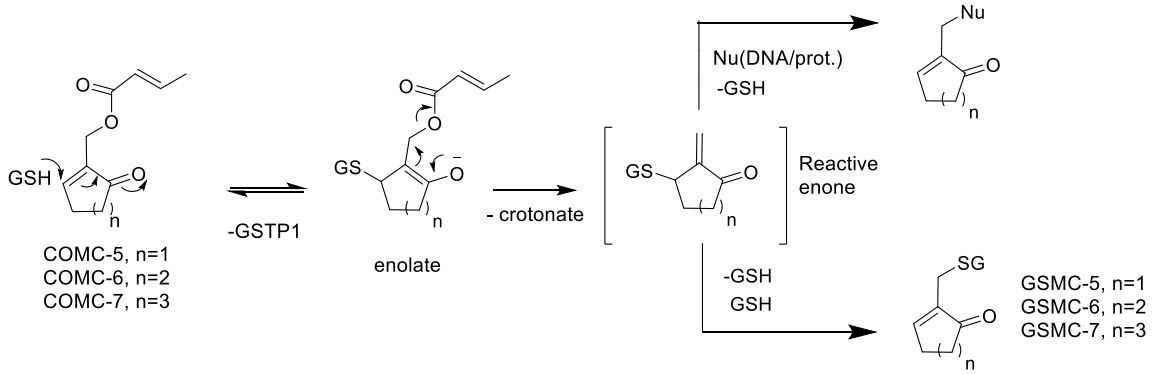
The same results occur with a synthetic analogue of COTC known 2-Crotonyloxymethyl-2-cyclohexenone (COMC)[87] This lead to the hypothesis that the biological activity is associated to the reaction into COMC and GSH to form an adduct (GSMC) by nucleophilic addiction and loss of crotonate portion (Scheme 1.3.),[88,89] however subsequent studies of IC_{50} in murine melanotic melanoma B16 cells excluded GSMC from being the cytotoxic species.[90]



Scheme 1.3. - Reaction of COMC and GSH to form the adduct GSMC

Then, kinetics studies indicated that these adducts formation implicates a multistep mechanism involving an electrophilic exocyclic enone intermediate into GSH and COMC/COTC by a Michael addition catalyzed by GST, and these electrophilic intermediate then reacts with a second equivalent of GSH to afford the GSMC.[90,91]

Indeed, under physiological conditions, the incubation of COMC with model dinucleotides or single-stranded 16-mer oligo-nucleotides, showed the formation of adducts of the exocyclic amino group of the guanine residues,[90,92] it means that the exocyclic enones can be likely to react with nucleophilic groups on nucleic acids (DNA) and proteins inside cells and DNA alkylation, for example, provides a plausible mechanism of cytotoxic activity, which is shared by a large number of chemical carcinogens and cross-linking antitumor agents (Scheme 1.4.).[92]



Scheme 1.4. – Proposed mechanism of a reactive enone formation and possible adducts formations from the enone

Moreover, GSTP1 are overexpressed in tumor cells when in presence of cytotoxic agents.[92] Kinetic studies indicate that the exocyclic enones require GSTP1-1 to be formed. In absence of enzyme the reaction of COMC-6 with excess GSH follows a first-order decay, showing no evidence of an intermediate species. Based on this, correlation of the GSTP1-1 expression level to cytotoxicity of the cyclic enones, were evaluated by incubation of cytotoxic agents with MCF-7piGST and MCF-

7wt breast tumor cells (the MCF-7piGST overexpress the pi GST isozyme at levels 10-fold greater than in wild-type tumor cell line).[92]

All of these results show that cycloalkenones transformed by GST to high electrophilic enones (capable of alkylating critical biomacromolecules) are of considerable interest as possible means of inhibiting MDR tumors, which often overexpress specific isozymes of GST.[93]

1.7. Promiscuity of Michael Acceptors

It is known that there is specificity for substrates that react with Michael acceptors (as cyPG). This specificity depends on the type of cell to act, the amount of GSH available, the side groups present on the CP ring, and, of course, the reactivity of the substrate that is influenced by the acquired conformation.[94,95]

Nevertheless, the cyclopentenones exhibit high reactivity, and due to the large number of targets available for alkylation, it may represent a problem for its application to the level of medicinal chemistry.

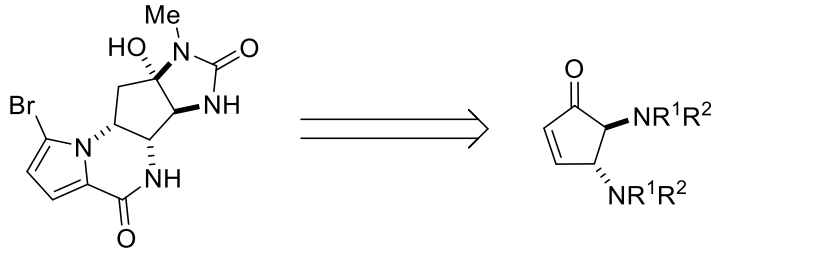
Thus, the presence of reactive enones in human cells (both in normal and cancer cells) may lead to severe side effects. In other words, Michael acceptors are considered Pan-assay interference compounds (PAINS) because they tend to react with numerous biological targets rather than the desired targets.[23,96,97]

Fortunately, studies of structure-activity relationship raise important questions about the ability to convert certain PAINS to NON-PAINS without loss of function. In our case, modifying the structure of compounds and consequently their behavior.

1.8. Synthesis of diamino cyclopentenones

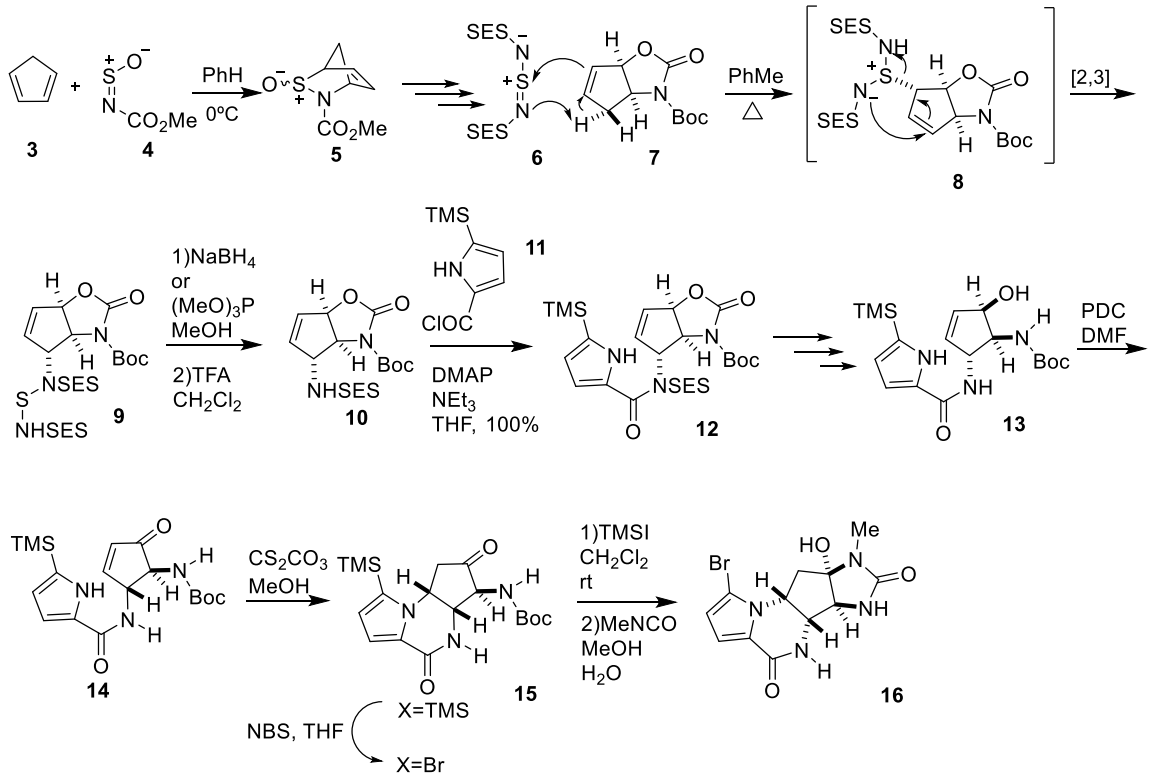
As mentioned above, CPs have been attracted attention of medicinal chemists. CPs as *trans*-4,5-diamine cyclopentenones could be employed as very useful intermediates towards antitumor natural products.

One example is the cytotoxic tetracyclic alkaloid known as (-)-Agelastatin A **1**, isolated from the axinellid sponge *Agelas dendromorpha* by Ambrosio *et al*,[98] which actuates as anticancer compound exhibiting potent cytotoxicity against L1210 leukemia in mice and human KB nasopharyngeal tumour cell lines.[99] This interesting biological activity coupled with the heterocyclic array contained in this type of alkaloids, makes them attractive targets for total synthesis, and their retrosynthetic analysis could lead to *trans*-4,5-diamine cyclopentenones **2** (Scheme 1.5.).



Scheme 1.5. – Retrosynthesis of Agelastatin A lead to *trans*-4,5-diamine cyclopentenones

The first total synthesis reported for **1** achieved a yield of 7% starting from Hetero Diels-Alder cycloaddition of N-sulfinyl methyl carbamate with cyclopentadiene which is the precursor of the carbocyclic C-ring, and has been reached in about 14 steps and performed in 12 operations by Stien *et al*. The key steps are the initial cycloaddition, followed by Sharpless/Kresze allylic amination, internal Michael cycloaddition of a pyrrole nitrogen to a cyclopentenone, a D-ring annulation of the bromopyrrole, and an addition of methyl isocyanate to an R-amino ketone (Scheme 1.6.).[100]



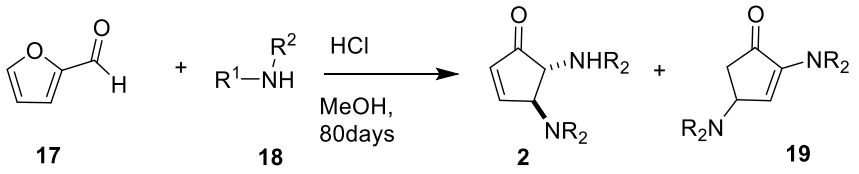
Scheme 1.6. – First total synthesis reported for Agelastatin A

Since then, many research groups have reported different synthetic pathways for Agelastatin A, and consequently for amino cyclopentenones, because it's one important precursor for Agelastatin, as explained above.

Among pathways we can highlight the methods by: Feldman *et al.,* which reported the first enantioselective syntheses involving 14 steps from (R)-epichlorohydrin where the most important transformation was a sulfinate-promoted cyclization of an alkynyliodonium salt to get a functionalized cyclopentene intermediate;[101,102] Hama *et al*, which involved 20 steps (yielding only 1%) starting with a sequential sigmatropic rearrangement followed by a cyclisation via ring closing metathesis to give *trans*-4,5-diamino cyclopentenol and after the *trans*-4,5-diamino cyclopentenone precursor;[103] Davis *et al*., which involved 11 steps, 23% yield, and was based in addition of a lithium enolate of ethyl (dibenzylamino)acetate to an acrolein-derived N-sulfinyl imine to give a sulfinimine-derived by 2,3-diamino ester followed by ring-closing metathesis to get 4,5-diamino cyclopenten-2-enone;[104,105] Hale *et al*, which started with acylation, hydrolysis and oxidation of a quiral oxazolidinone to give a diamino cyclopentenone (afforded after 5 steps with an yield of 14%), followed by intramolecular Michael addition with the cyclopentane rings and a pyrrole and finally hydrogenation and monobromination.[106,107] Other total synthesis should be highlighted such as from Dikson *et al*, Trost and Dong's *et al*.

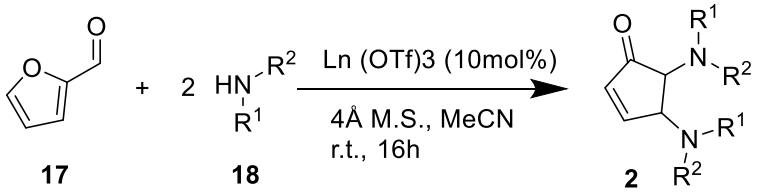
Besides the enormous advances in this area, the reported total synthesis still has some limitations such as the high number of steps and overall low yields. For these reasons the development of one method for a small and efficient synthesis would be highly useful.

In 1850, was reported by J. Stenhouse the formation of salts named Stenhouse salts (SSs) resulting from the furan ring-opening reaction upon condensation of furfural **17** and amines **18**.[109-111] After that, other studies such as the one from Lewis et al. revealed that these SS under harsh conditions (refluxing methanol in the presence of hydrochloric acid) undergo a thermal 4π-electrocyclization reaction to give the colorless 4,5-diamino-2-CP, however this reaction was not recognized for many years because **2** could rearrange into the thermodynamically more stable 2,4-diamino-CP **19** (unselective reaction). Allso because the reaction times are long (80 days) and the yields poor (Scheme 1.7.).[112-116]



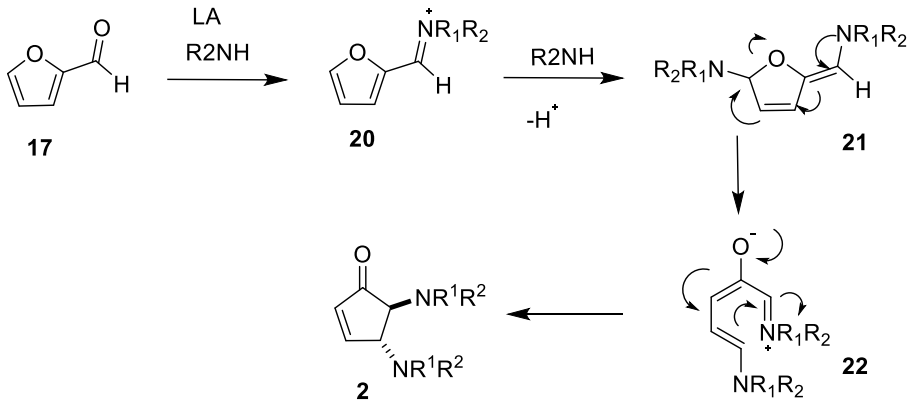
Scheme 1.7 – Synthesis of *trans*-4,5-diamino cyclopentenones from furfural and anilines. Lewis *et al* method.

Trying to get around this trouble, Li and Batey reported a protocol to the formation of *trans*-4,5-diamine cyclopentenones from furfural and secondary amines. Initially was used protic solvents such EtOH, in the presence of excess catalyst such $BF_3.OEt_2$ $Ti(OiPr)_4$, $Al(OiPr)_3$ and $B(OMe)_3$. Were obtained good yields, instead of the absence of Lewis acids or the presence of protic acids like HCl, but curiously for the lanthanide Lewis acids (prominently $Dy(OTf)_3$) the reaction proved to be very efficient (with excellent yield and excess diastereomeric > 95%) in MeCN (Scheme 1.8.). They have also analyzed the formation of these cyclopentenones reacting **17** with primary amines, but all the reactions were unsuccessful, except for aniline.[117]



Scheme 1.8. – *Trans*-4,5-diamine cyclopentenones from furfural and secondary amines. Li and Batey Method

A mechanism has been proposed for this reaction. It starts with the formation of an iminium ion which would undergo addition in position 5. Subsequent furan ring-opening affords the deprotonated Stenhouse salt **22** that after Nazarov electrocyclization yields the CP **2**. NMR studies to the cyclisation of the Stenhouse salts indicated a fast ring closure (first order kinetics) and computational studies showed that the ring-closure is consistent with a thermal conrotatory 4π electrocyclization suggesting a Nazarov mechanism and the *trans* stereochemistry is consistent with the Woodward-Hoffmann rules (Scheme 1.9.).[117]

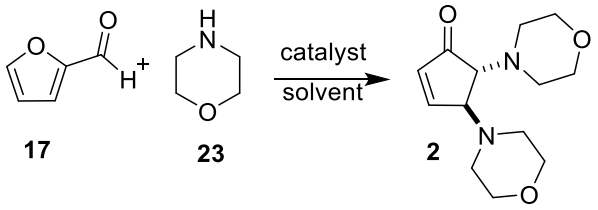


Scheme 1.9. – Proposed mechanism for *trans*-4,5-diamine cyclopentenones synthesis by Li and Batey.

After this pioneer work by Li and Batey, other protocols has been reported such as from Ramesh's group which the reaction is catalysed by the acidic ionic liquid 1-methylimidazolium tetrafluoroborate [Hmim]+[BF_4] and does not require anhydrous conditions neither solvent because the IL plays the dual role of catalyst as well as recyclable reaction medium; [118] from Procopio's group, where the catalytic activity of Er(III) was tested using Er(OTf) $_3$, in acetonitrile and Ethyl Lactate at different experimental conditions, and then using $ErCl_3$ and $ErCl_3 \times 6H_2O$ which is moderately expensive and has low toxicity;[119] from Wang's group which the method is catalysed by tosylamine at high temperatures (80ºC) in acetonitrile during 5h;[120] from Kostakis's group where the reaction is catalysed by DyIII/NiIII heteronuclear clusters in acetonitrile[121,122] and the protocol from Nardi's group that is an environmentally friendly method in water under microwave irradiation at high temperatures (60 °C) (Table 1).[123]

Our group have been also involved in methods to prepare **2**. One of these involves the use of erbium(III) chloride immobilized on silica as a reusable catalyst, proving to be a very efficient catalyst in n-butanol at room temperature and mild reaction conditions.[124] Another reported method involves the use of erbium(III) chloride hydrated in ethanol, at 50ºC and mild conditions during 30 min. and is considered suitable for use in introductory organic chemistry laboratory classes.[125] The last reported method from our group is considered environmentally friendly owning to the use of copper(II) triflate in water, at mild reaction conditions with great efficiency (Table 1).[126]

Table 1 - Reported methods for the preparation of 4,5-diamino-CPs basing in Li and Batey Method.



17 23 2

Previous work	Catalyst / solvent	Yield
Li and Batey, **2007**	$Dy(OTf)_3$ / acetonitrile	quant. (16 h)
Hamesh, **2009**	$[HMim][BF_4]$	98% (5 min)
Procopio, **2013**	$ErCl_3.6H_2O$ / ethyl lactate	99% (30 min)
Wang, **2013**	$TsNH_2$ (80ºC) / acetonitrile	86% (5h)
Kostakis, **2015, 2016**	Dy^{II}/Ni^{II} cluster / acetonitrile	quant. (16 h)
Afonso, **2017**	$ErCl_3.6H_2O$ on silica / n-butanol	66% (30 min)
Afonso, **2017**	$ErCl_3.6H_2O$ / ethanol	88% (30 min)
Nardi, **2017**	MW (60%) / water	quant. (5 min)
Afonso, **2018**	$Cu(OTf)_2$ / water	quant. (1 min)

1.9. Synthesis of 2,4-bifunctionalised cyclopentenones

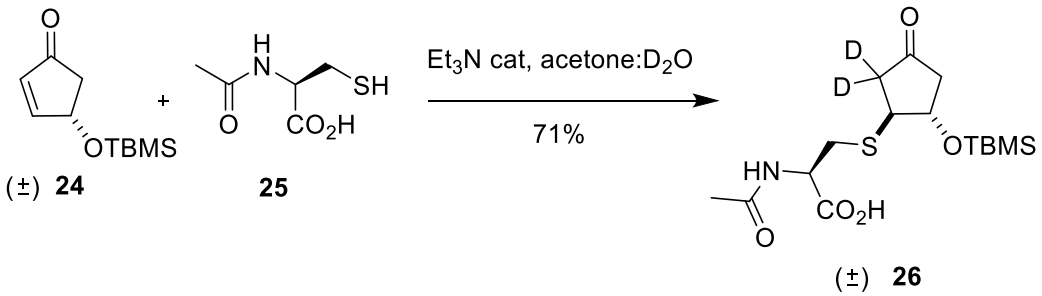
The diamino-cyclopentenones are Michael acceptors, and as we explained above, can react with nucleophiles. Two types of addictions may occur: conjugated (1,4 addition) or direct (1,2 addition). The thiols usually react with CPs by conjugated addition. This can be explained by the type of attraction between electrophiles and nucleophiles. Thiol is a soft nucleophile and β-carbon of CP is a soft electrophile (because they have larger atoms with diffuse orbitals), and soft molecules tend to be attracted to with each other by a dominant orbital control.[23]

Some protocols that highlight the reactivity principles of 1,4 additions to thiols have been reported.[127-132]

One of these, studied the reaction of cysteine derivates with prostaglandins (PGA1) in order to understand their mechanism. Suzuki's group showed that the conjugation was selective to the endocyclic double bond, even were another exocyclic double bond in a position was also in conjugation with the carbonyl group, and this conjugation to the exocyclic bond only took place in presence of higher amounts of thiol, at longer times and when the other was already thiolated. It was also observed that the stability of the formed adduct was inversely proportional to pH.[127]

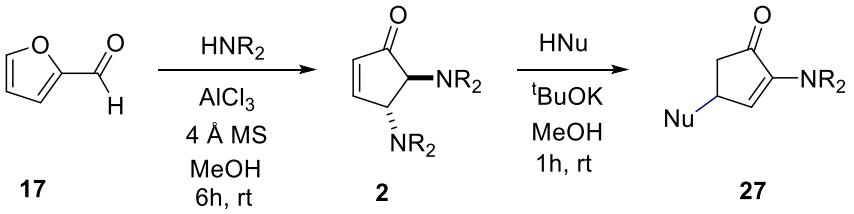
Bickley's group using cyclopentenones with cysteines observed that thiols added exclusively *anti* regarding to OTBDMS on the third position to give *trans* product **26** and was observed reversibility of the addition when exposed to physiological pH (scheme 1.10).[128] The anti diastereoselectivity was also observed in the synthesis of an aristomycin analog by *Das et al*,[129] and Dauvergne's group also used a *trans* thioadduct (formed in good yield) like intermediate to the replacement of C-4 tosylate in studies about the functionalization of cyclopentenones.[130]

Despite the soft character of the thiols, the thiolation can be a very sensitive procedure. This was proved in the synthesis of Mannostatin A and analogs by Cho *et al.* when aminodiol diastereoisomers afforded different products.[131]



Scheme 1.10 – Bickley's method for thiols addition to cyclopentenones

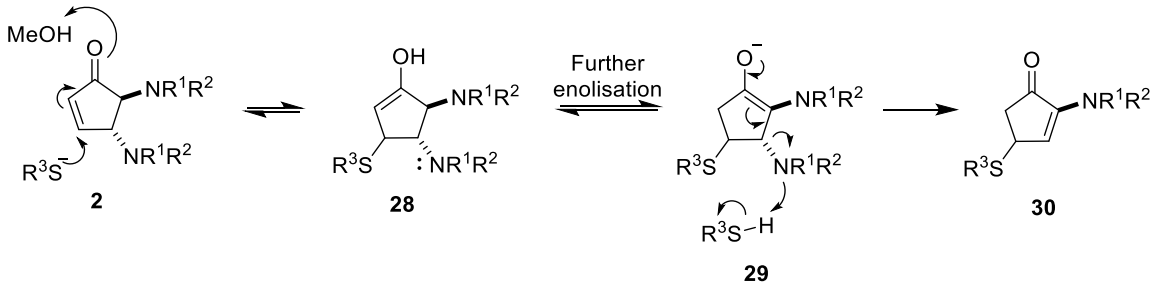
Basing on the method from Li and Batey, our group in 2013 described a one pot method of synthesis of 2,4-bifunctionalised cyclopentenones via conversion of 2-furaldehyde **17** with morpholine **23** followed by concomitant 1,4 addition (Scheme 1.11). In the optimization of this synthesis was observed that the use of protic solvents such as MeOH improved reaction rates and yields, and most hard oxophilic Lewis acids were effective. After some screening, the optimal Lewis acid chosen to catalyse the formation of **2** was $AlCl_3$ (0.1 eq.) and the base for conjugated addition and formation of **27** was KO^{t}Bu (0.25eq.) The reaction of the diamine CP intermediate with alkoxides (nucleophiles), showed that the excess of these affords undesirable side products by the competition of conjugate addition into alkoxide and morpholine. Then, this method was applied for amines and thiols.[132]



Scheme 1.11 - Synthesis of 2,4-bifunctionalised cyclopentenones by a one-step reaction.

Combining all evidences found at the optimization process, was possible to understand the mechanism of the reaction. It proceeds by 1,4 addition of thiol to the diamino cyclopentenone **2** followed by E1cB elimination (Scheme 1.12).[132]

The E1cB elimination (**E**limination **U**nimolecular **c**onjugate **B**ase) is a reaction in which two groups are eliminated, a poor leaving group on the α-carbon and an acidic hydrogen on the relatively β-carbon under basic conditions. It starts with the abstraction of the most acidic proton by the base generating a stabilized enolate (the conjugate base or anion of enol) followed by the elimination of the leaving group and formation of double or triple bond (because the lone pair of electrons on the anion moves to the neighbouring atom).[133]

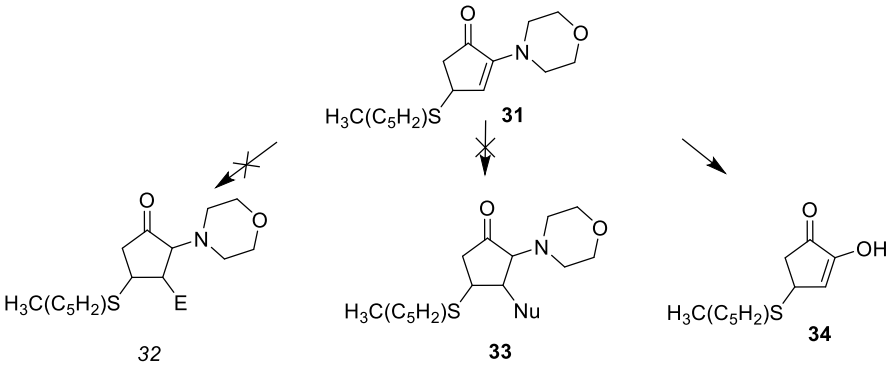


Scheme 1.12 – Proposed mechanism for the synthesis of 2,4-bifunctionalised cyclopentenones

1.10. Further transformations of 2-morpholino-4-thioalkyl-cyclopentenones

Our group has speculated if these 2-morpholino-4-thioalkyl-cyclopentenones **30** could be transformed into new products by reacting them toward electrophiles and nucleophiles.

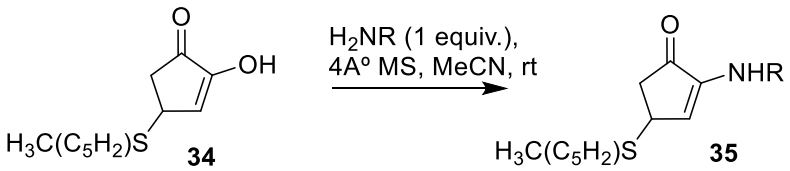
First, was attempted react with CP **31** electrophiles such allyl bromide, p-fluorobenzyl bromide and p-chlorobenzyl bromide in toluene and MeCN, at different temperatures for prolonged periods of time, and there was no reaction. Only in presence of base such DBU and Et_3N at high temperatures over a few hours or days was observed partial reaction but was afforded more than 3 products hard to isolate with very low yields (Scheme 1.13.).[132] Then, was also attempted react **31** towards nucleophiles such α-toluenethiol and thiophenol in MeCN and toluene, at different temperatures over several days, under normal and basic conditions. And there was also no reaction but in acidic conditions using PTSA monohydrate was observed the formation of an hydrolysis product (Scheme 1.13.).[132]



Scheme 1.13. - Illustration of the reactions of 2,4-amino-thio cyclopentenones towards electrophiles, nucleophiles and acids

After optimization was found that the mixture of MeOH:H_2O (4:1) was sufficient (the absence of water gave multiple products) and HCl was a suitable acid for the reaction and that its excess could afford undesirable products. Thus the optimal conditions for hydrolysis stablished are: HCl (1.1 equiv.) in of MeOH:H_2O (4:1) at 60ºC for 2 hours. [132]

Once primary amines could not be condensed with 2-furaldehyde, these could be easily introduced onto hydroxy cyclopentenenone compounds with moderate yields, however aniline and secondary amines were difficult to insert (Scheme 1.14.).[132]



Scheme 1.14 - Reaction into hydroxyl-CP and primary amines

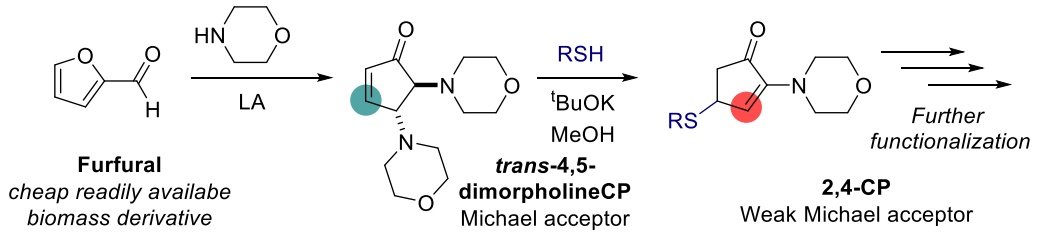
2

Objectives

Due to the unsaturated α,β-carbonyl group of cyclopentenones, these compounds act as Michael acceptors and may react with various nucleophiles making them dangerous compounds (PAINS).[23]

Thus, the objective of this work is the development of a small family of CP with very poor Michael acceptor character that possess cytotoxic activity for HT-29, MCF-7 and NCI-H460, respectively colon, breast and lung cancer cells (which are currently the most frequent types of cancer in the world).[17] It means that they could retain the cytotoxic activity through a mechanism of action different to the non-specific macromolecule alkylation, and it could avoid severe side effects.

Our group has been working on the preparation of *trans*-4,5-diaminocyclopentenones from furfural (cheap furan derivative, easily available and obtained from non-edible carbohydrates such as xylose). From these compounds was observed the addition of a thiol to the enone under basic conditions, followed by consequent elimination of an amine to reestablish the CP and was also observed no second addition of the excess thiol to this newly formed enone, which makes it poor Michael acceptor. Therefore, is important to synthetize new CPs with cytotoxicity to cancer cells and to prove its poor Michael character by interaction for example with glutathione (Scheme 2.1.)



Scheme 2.1. – Illustration of this work reactions

3

Results and Discussion

3.1. Synthesis and Biological Assays

3.1.1. *Trans*-4,5-morpholine cyclopentenone

We have synthetized *trans*-4-5-morpholine cyclopentenone by two different methodologies.

The first method is based on the one-step reaction before proposed and reported by our group, but the difference is that now we divide the reaction in two steps: (1) synthesis of *trans*-morpholine cyclopentenone **42** and 2) nucleophilic conjugated addition, because we needed large amounts of **42** to react it with several nucleophiles.

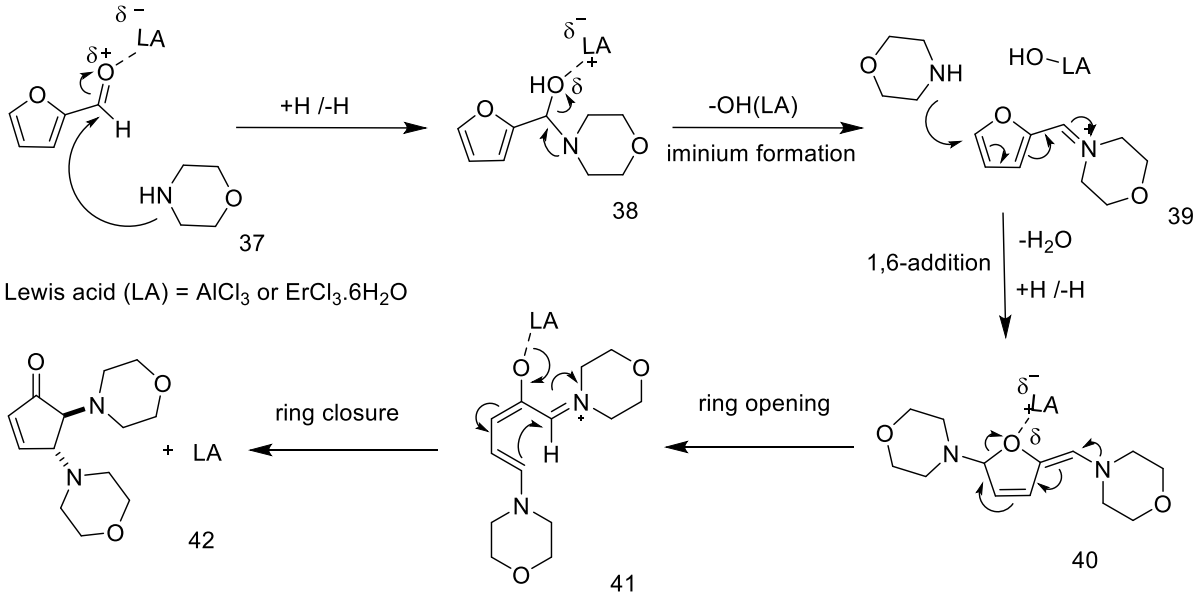
Thus, we prepare **42** by reacting two equivalents of morpholine (a secondary amine) with furfural in MeOH (a protic solvent), in the presence of $AlCl_3$ at room temperature under inert atmosphere. In order to achieve a successfully reaction the elimination of water was essential inside the reaction medium (for that we used $MgSO_4$ that is a fast drying agent).

This reaction gave excellent yields (94%) after work up (filtration and extraction), and the product **42** did not need to be further purified. Usually a yellow solid is formed but when this does not happen by itself it is necessary to employ the addition of a small amount of diethyl ether, slightly polar solvent, because it is good for crystallization. The work up that consists by filtration, addition of Brine and extraction to the organic phase with DCM, is important to eliminate the metal to the reaction.

The other method used to synthetize **42**, is quite similar to the previous one but the catalyst used was $ErCl_3.6H_2O$ and the solvent was acetonitrile. This method also shown to be effective affording excellent yield (92%) and it has some advantages when compared to the other: inert atmosphere is not necessary, and the reaction is complete in 12 times shorter time (30 min.). The

strictly anhydrous conditions for the first approach are essential because the $AlCl_3$ is a Lewis acid catalyst that immediately reacts with water rather than with substrates and stops the reaction. On the other hand, in case of $ErCl_3.6H_2O$ the presence of water may be beneficial.[134]

The proposed mechanism for these reactions are shown below in scheme 3.1.



Scheme 3.1. – Proposed mechanism for *trans*-4,5-morpholine cyclopentenone from furfural and morpholine

The reaction starts with the condensation (catalyzed by the Lewis acid) of 2-furaldehyde and one molecule of morpholine to form the iminium ion intermediate **39** that is unstable. Previous experimental observations and computational studies showed that its formation is the rate limiting of the mechanism. Then, occurs a second condensation of another molecule of morpholine with the iminium ion at the 5-position of furan ring by 1,6-addition that promotes the furan opening to yield the deprotonated Stenhouse salt **41** which can undergo conrotatory 4π electrocyclization to yield the final *trans*-4,5-diaminocyclopent-2-enone derivatives as a single diastereoisomer.

The structure of the compound was confirmed by NMR spectroscopy and agrees with those described in literature. It is possible to observe the protons of the double bond represented by doublets of doublets with identical coupling constants, being that the one closest to the more electronegative heteroatom appears in a more deshield region (greater chemical deviation). The

protons of morpholines are in an aliphatic region (2.5-3.75ppm) and are characterized by several signals with different unfolding (Figure 3.1).

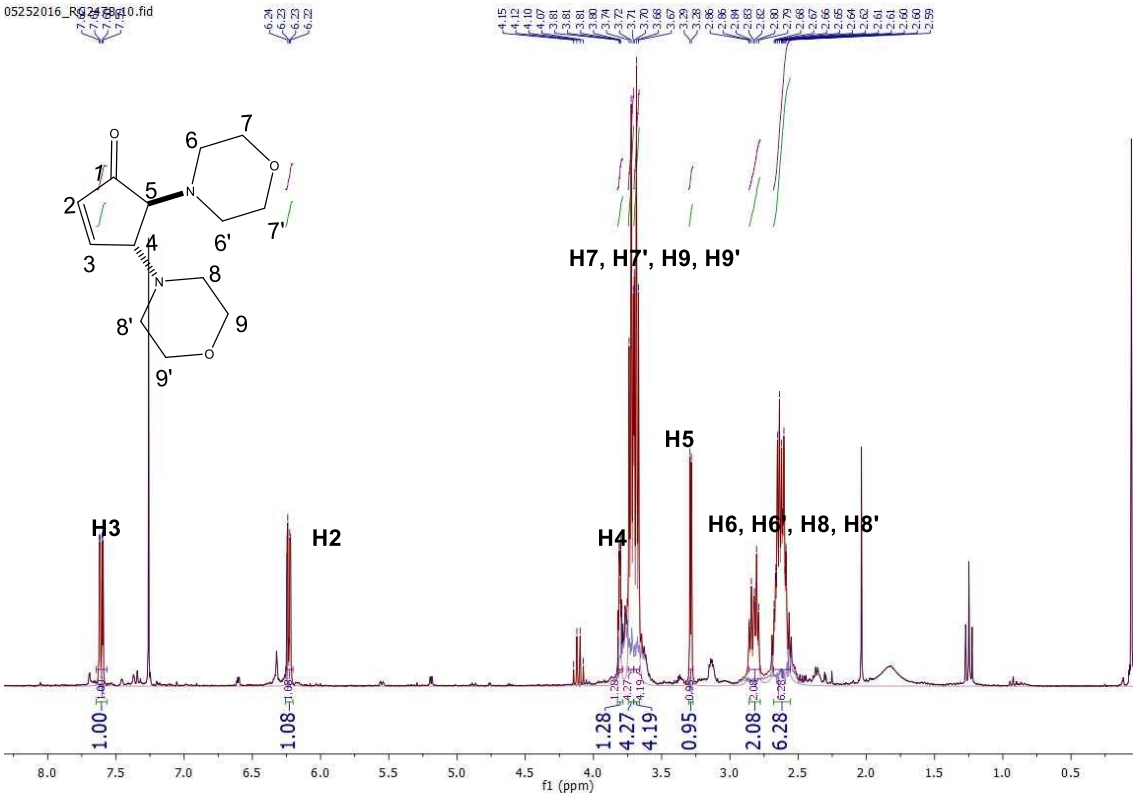


Figure 3.1 –NMR spectrum of *trans*-4,5-dimorpholinocyclopent-2-en-1-one

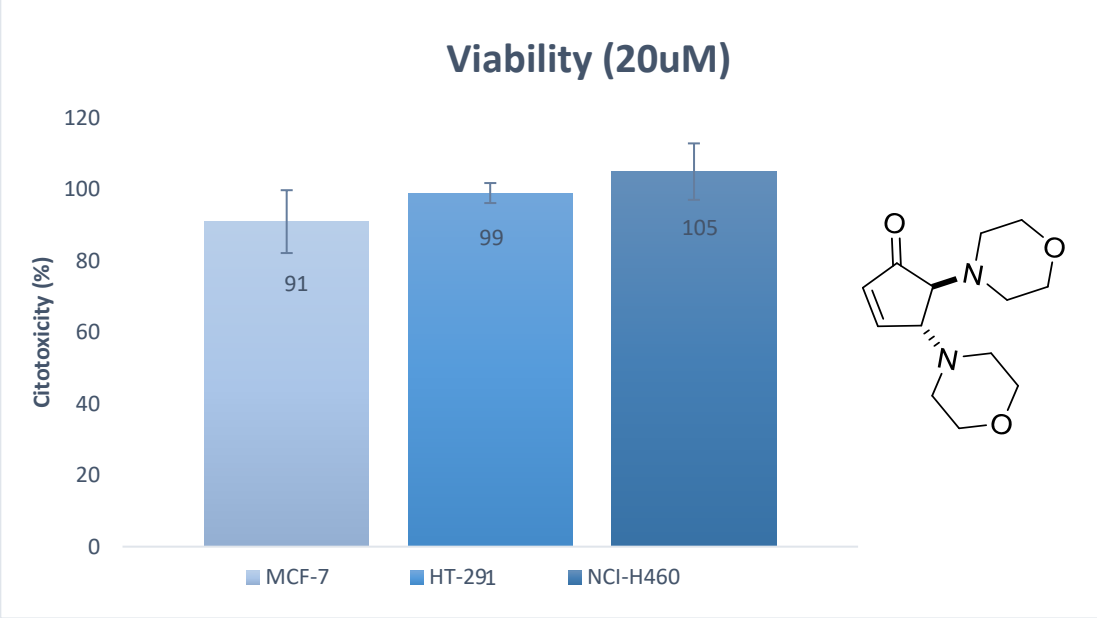
Once synthesized, this compound was subjected to cytotoxic assays in HT-29, MCF-7 and NCI-H460. The cytotoxicity can be measure by calculation of IC_{50} that is, in our case, the concentration of compound required for 50% inhibition of the biological process that inhibits apoptosis, it means that is a measure of how effective the compound is to lead to cell death.

To that end, the cells were seeded in 96 well plates at a suitable concentration, depending on the cell line, and 24h later the compound (previously dissolved in appropriate cell DMSO and diluted in culture medium supplemented with fetal bovine serum and antibiotic solution) was incubated with them for 48 hours. Then, they were treated with a Neutral Red dye which absorbs at 540nm and the viability was then measured by spectrophotometry.

The culture medium (RPMI, complex synthetic medium) is a mixture of salts enriched with essential components for cell growth. Fetal bovine serum contains a large number of essential components, not found in the medium, such as fatty acids, amino acids, vitamins and growth factors whose function is to promote cell growth and is obtained from coagulated blood, and the antibiotic and antimycotic has the purpose of preventing contamination with fungi and bacteria.

For calculations and hence determination of cytotoxicity a control only with DMSO diluted in medium (at the same concentration present in the wells with compounds) was required. The measured absorbance values for the compound are divided by the absorbance value of the DMSO well.

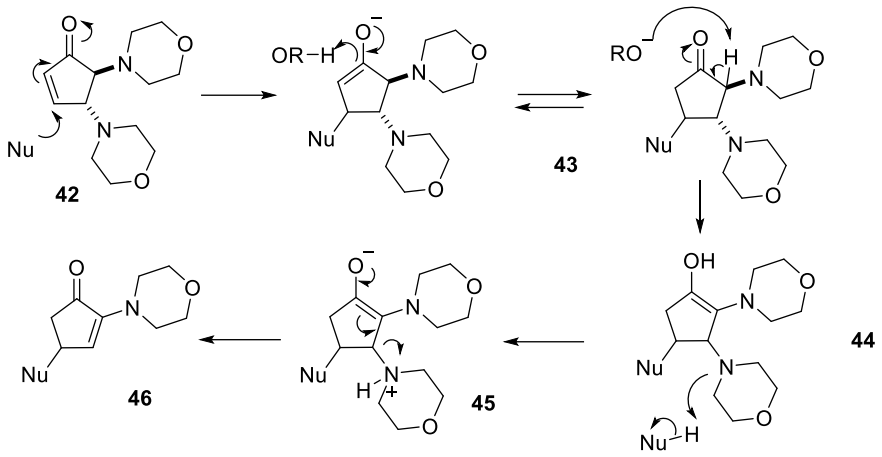
For this first compound were performed assays at 20µM. If the percentage of cytotoxicity is < 50% for 20µM, we consider that this molecule will have a good value of IC50 (<20µM) comparing to other cytotoxic compounds. For this diamine CP 42 the percentage of viability was high, it means that it is a poor cytotoxic compound (Graph 1.).



Graph 3.1. - Values of cellular viability obtained after exposure of the MCF-7, HT-29 and NCI- H460 tumour cells to *trans*- 4,5-morpholine cyclopentenone at 20 µM, for a period of 48 hours. Viability was determined with the Neutral Red reagent and the experimental points represented are the mean of 3 replicates per experimental condition and the experimental error is the standard deviation.

3.1.2. 2,4-bifunctionalised cyclopentenones

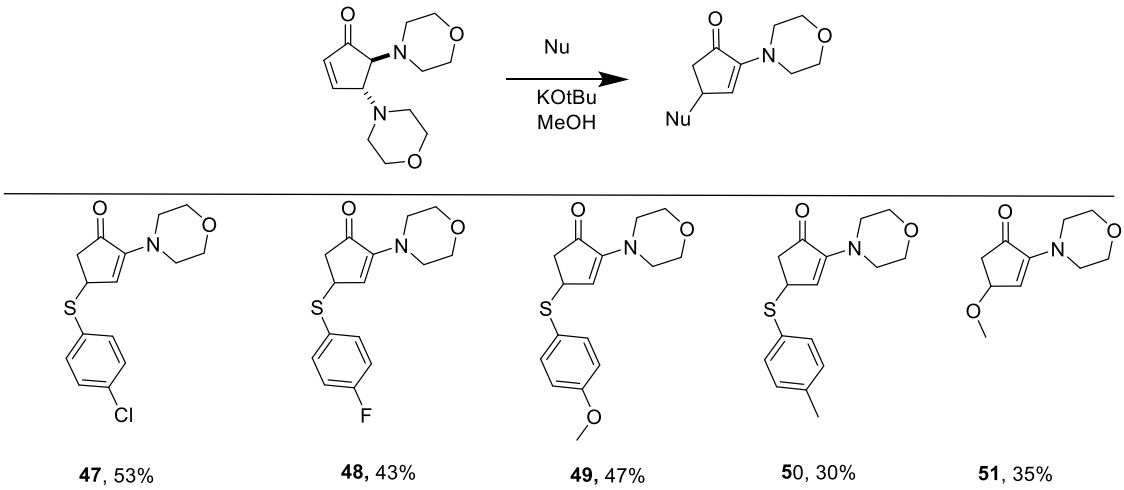
The desired poorly electrophilic CPs could be obtained by reacting **42** with a nucleophile in the presence of KO^{t}Bu using methanol as solvent. The mechanism of the reaction is shown below in scheme 3.2**.**



Scheme 3.2. – Proposed mechanism for the synthesis of 2,4-bifunctionalised cyclopentenones from *trans*-4,5-morpholine cyclopentenone

It starts with the 1,4-addition of the nucleophile to the electrophilic centre of **42**, forming an enolate (the negative charge is stabilized by resonance with the adjacent carbonyl group) which are protonated by the MeOH leading to a neutral product. Then, the morpholine at C4 of **44** are eliminated by E1cB reaction: the proton is abstracted from the C5 generating a stabilized anion, the lone pair of electrons moves to the neighbouring rotes and the morpholine at C4 (previously protonated most likely by another molecule of nucleophile) are eliminated, generating the enone **46**.

The reaction of **42** with nucleophiles such as thiols, for example, is an excellent method to obtain poor Michael acceptors, because the electrophilic centre that usually reacts with critical macromolecules to cells is blocked (Scheme 3.3.).



Scheme 3.3 – Above, the generic scheme for the synthesis of 2-morpholine-4-substituted CP in MeOH at basic conditions. Below, the molecular structures of 2,4-bifunctionalised cyclopentenones synthetized

The structure of these compounds was confirmed by NMR spectroscopy experiments, proving that they were successfully isolated with high purity (Appendices). Below, we present the spectrum of the compound **47** to exemplify. In ^{1}H NMR spectrum (Figure 3.2), the morpholine moiety is represented by the signals H6, H6' (multiplet) and H7, H7' (triplet) appearing in the aliphatic region while the chlorothiophenol by H9, H9' and H10, H10' appearing in the aromatic region, but overlapping because they couple with each other. Beyond the signals of the aromatic protons, the presence of the C5 geminal protons (characterized by two doublets of doublets) and of a proton bound to the vicinal carbon atom at thiol, allows us to state that the 1-4-addition of morpholine occurred. We still have the presence of the proton of the double bond that appears practically to the same chemical deviation that in the spectrum of compound **42,** this proton confirms the elimination of one morpholine molecule.

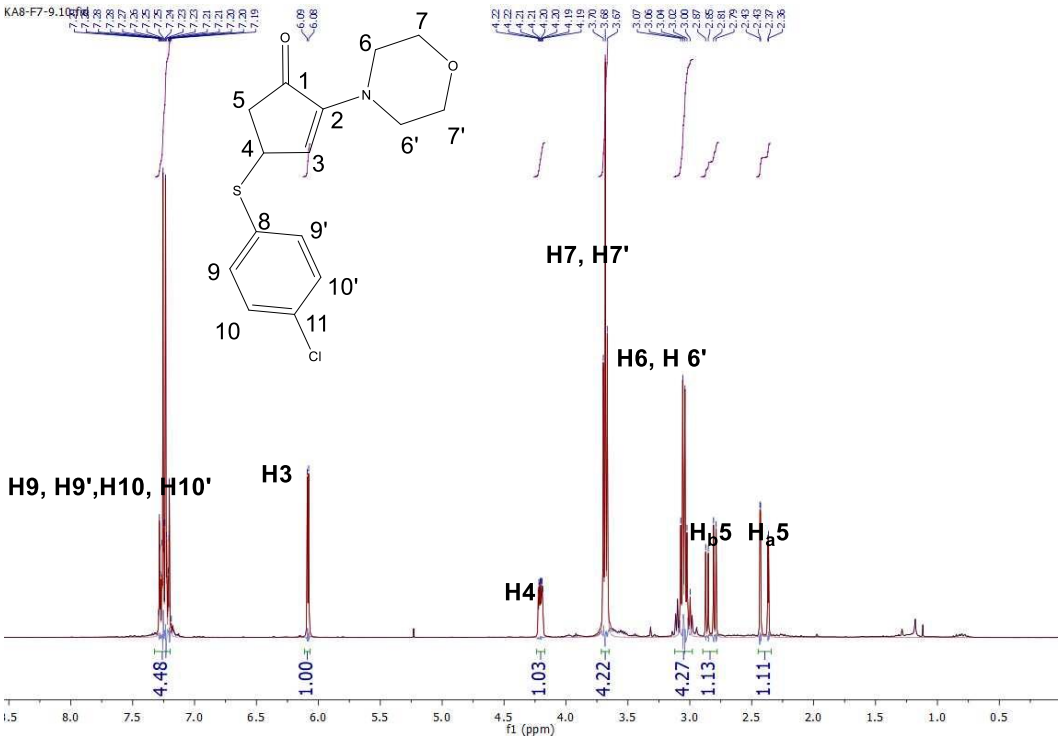
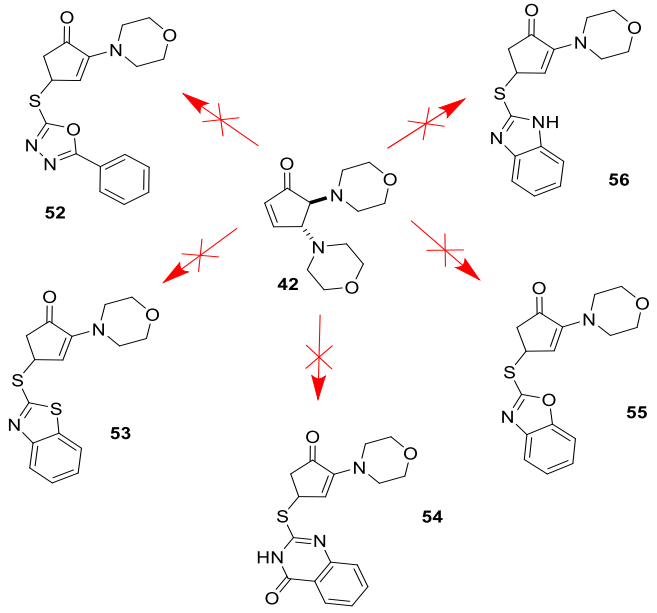


Figure 3.2 – NMR spectrum of 4-((4-chlorophenyl)thio)-2-morpholinocyclopent-2-en-1-one

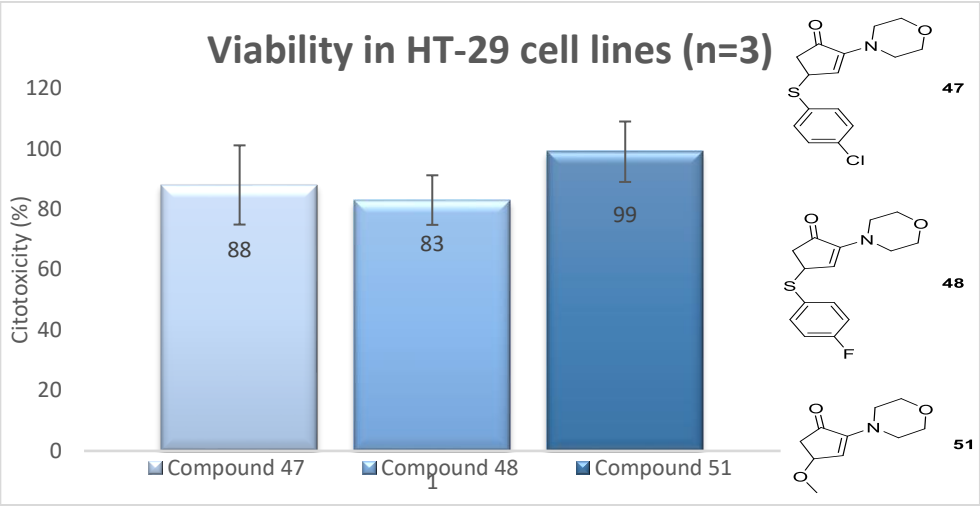
The spectra of the other synthesized compounds are very similar, because they differ only in the signals of the substituents. Relative to the spectrum of compound **48**, Fluor influences the values of the coupling constants of the aromatic protons which appear in 2 doublets of doublets. In the spectrum of compound **49,** a singlet for the protons of the methyl group in the aliphatic region appears, and in the spectrum of compound **50** also appears the singlet, but the protons are more deshield due to the proximity to the heteroatom. In the case of compound **51**, the singlet also appears, however the signals in the aromatic zone relative to the phenol no longer appear.

We also tried to react cyclopentenone with other thiols, however the reactions were unsuccessful. Many by-products were formed which even after purification were difficult to identify, because there were many peaks superimposed on the NMR spectra. These thiols are bulk and contain heteroatoms that could react to undesirable products (Scheme 3.4).

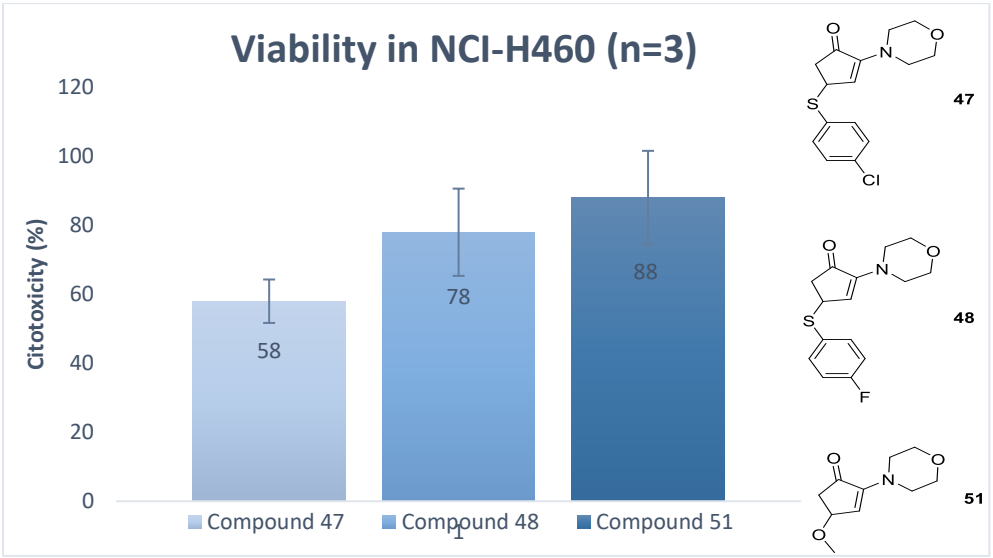


Scheme 3.4 – Unsuccessful attempts of 2,4-bifunctionalised cyclopentenones synthesis

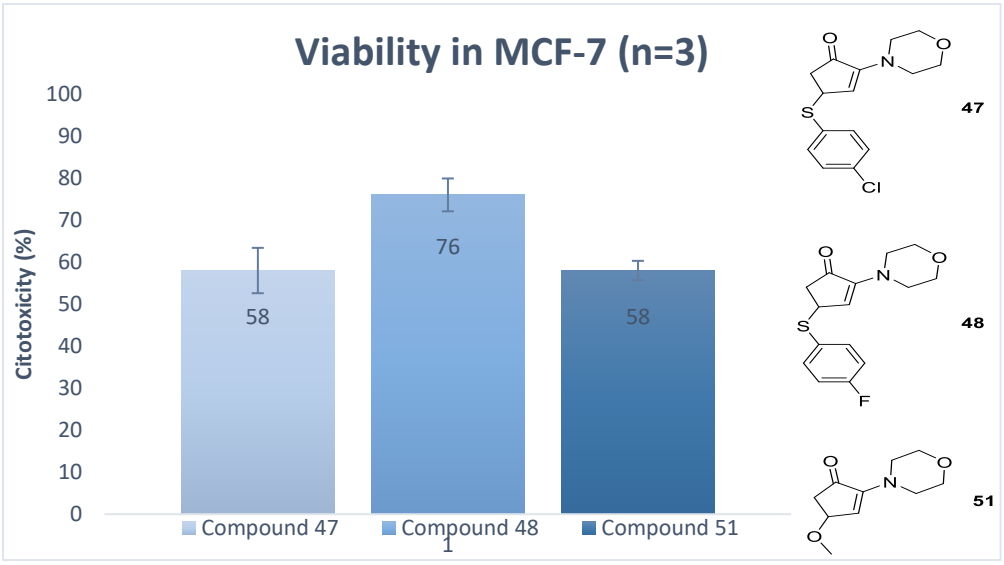
For the biological assays, the compounds tested so far were: **47**, **48** and **51** (Scheme 3.3). These were tested at a concentration of 20µM in triplicate in HT-29 (Graph 2), NCI-H460 (Graph 3) and MCF-7 (Graph 4), and showed higher cytotoxicity to breast tumour cells, such as *trans*-4,5-morpholine cyclopentenone. Standard deviations are acceptable, especially for breast cells that do not exceed 5%. Although the compounds show higher cytotoxicity for MCF-7 cells, we have not yet had significant viability results demonstrating that these morpholino-compounds are considerably cytotoxic.



Graph 3.2 - Values of cellular viability obtained after exposure of the HT-29 colon tumour cells to 2-thio-4-morpholine cyclopentenones at 20 µM, for a period of 48 hours. Viability was determined with the Neutral Red reagent and the experimental points represented are the mean of 3 replicates per experimental condition and the error experimental is the standard deviation

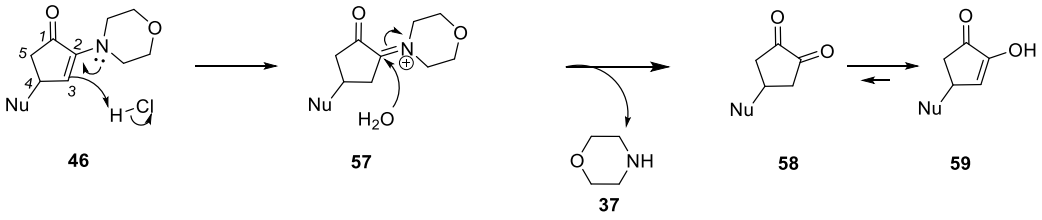


Graph 3.3 - Values of cellular viability obtained after exposure of the NCI-H460 lung tumour cells to 2-thio-4-morpholine cyclopentenones at 20 µM, for a period of 48 hours. Viability was determined with the Neutral Red reagent and the experimental points represented are the mean of 3 replicates per experimental condition and the error experimental is the standard deviation



Graph 3.4 - Values of cellular viability obtained after exposure of the MCF-7 breast tumour cells to 2-thio-4-morpholine cyclopentenones at 20 μM, for a period of 48 hours. Viability was determined with the Neutral Red reagent and the experimental points represented are the mean of 3 replicates per experimental condition and the error experimental is the standard deviation

We thought that hydrolysis of morpholine to form 2-hydroxy-4-substituted-CPs (2HCPs) would enhance the solubility in aqueous media and that the electrophilic character of the enone would decrease (even further since the olefin is in fact an enol) making these 2HCPs very poor Michael acceptors. These morpholine cyclopentenones are hydrolysed under acidic conditions (HCl 1.1 equiv.) in a MeOH: H_2O mixture at 60ºC and the mechanism of the reaction is showed on the scheme 3.5.

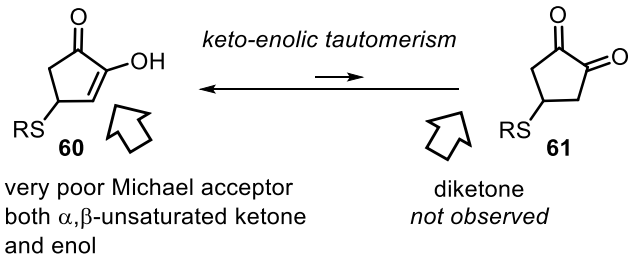


Scheme 3.5 – Proposed mechanism for the synthesis of 2-hydroxy-4-substituted-CPs from 2-thio-4-morpholine CP

The hydrolysis is catalysed by hydrochloric acid. First, the enamine picks up a proton from the acid, and the free electrons of the amine delocalize affording an iminium cyclopentenone **57**,

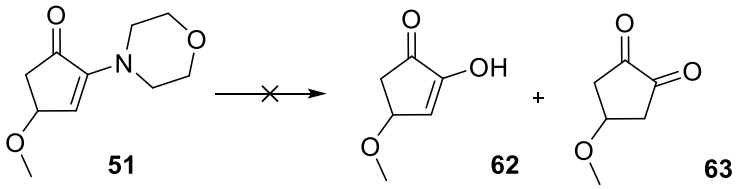
which can be attacked by water at C2, eliminating the morpholine. Thus, is formed a diketone **58** which tautomerize to an enol **59**.

At the reaction conditions, the diketone is not observed, possibly due the increased thermodynamic stability of the α,β-unsaturated system (Scheme 3.6).



Scheme 3.6 – Keto-enolic tautomerism of 2-hydroxy-4-substituted-CPs

Not all of the compounds synthesized in the previous step were hydrolysed. The attempt to hydrolyse compound **51** failed (Scheme 3.7).



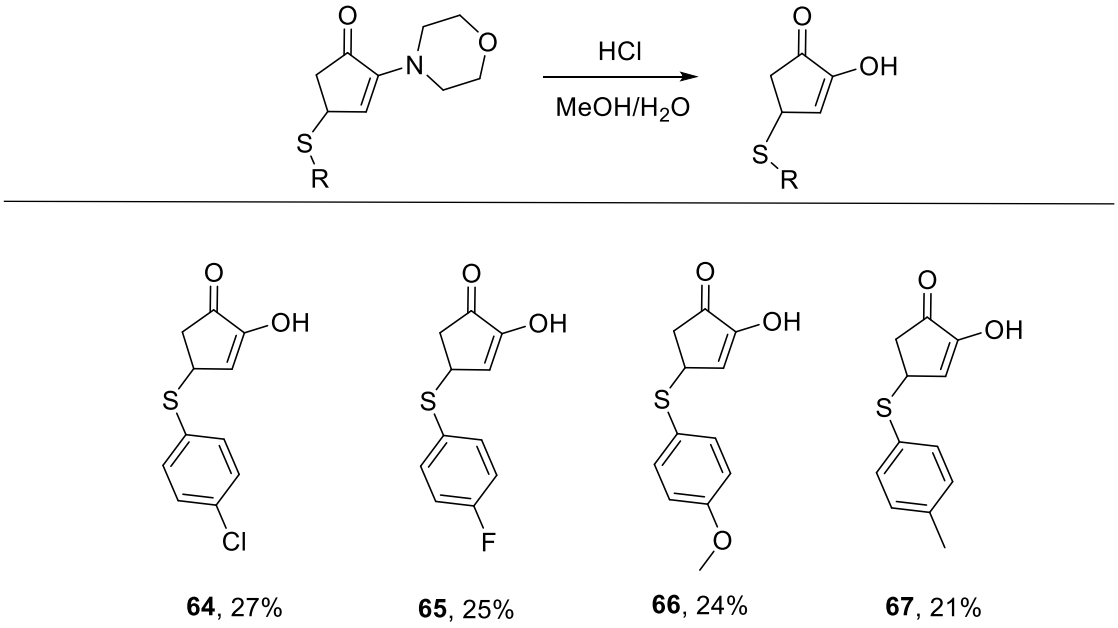
Scheme 3.7 – Illustration of the unsuccessful hydrolysis of **51**

The optimum time observed to these hydrolysis reactions was of 2 hours. At this time the percentage of product / starting material was approximately 50%: 50% in all studied compounds. The excessive reaction time and the excessive use of acid achieved undesirable products.

Thus, the yield could not be increased, so we then tried to purify the product by chromatography. However, the rf (retention factor) of the starting material and the product in the TLCs are so close that purification per column of silica, alumina even by preparative is inefficient. It has also been observed that the product degrades in the column, probably because it is a non-stable enol under acidic conditions. Next, we attempt to purify by precipitation.

Precipitation was not a very effective technique for us, but it was the best way we got. We attempted to precipitate with methanol and diethyl ether (changing proportions) by heating and cooling. Sometimes the starting material has precipitated, and the product has remained in solution,

and sometimes the other way round. Thus, we obtained pure product fractions, but most of the time, fractions are contaminated with a small percentage of morpholine cyclopentenone (Scheme 3.8).



Scheme 3.8 – Above, the generic scheme for the synthesis of 2-hydroxy-4-substituted CP in MeOH: H_2O under acidic conditions. Below, the molecular structures of 2-hydroxy-4-substituted-CPs synthetized

NMR analysis of these compounds is distinguished from previous ones mainly by the absence of the morpholine signals (between 3 and 4 ppm) and the presence of the singlet referring to OH (Figure 3.3).

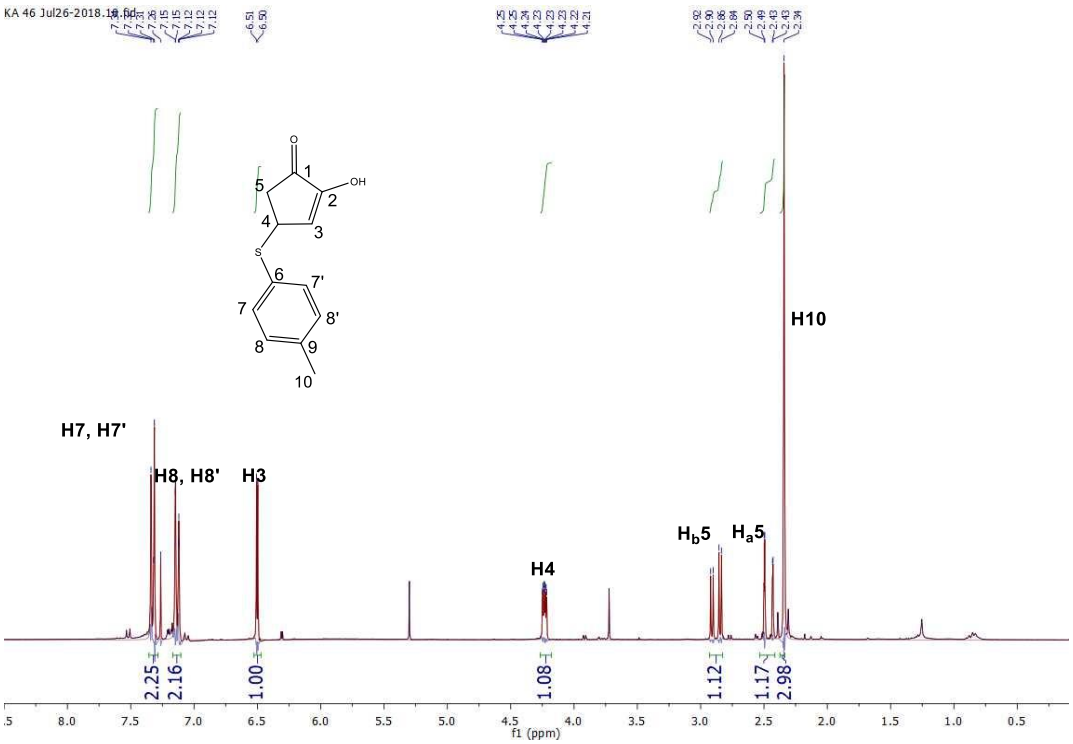


Figure 3.3 - NMR spectrum of 2-hydroxy-4-(p-tolylthio) cyclopent-2-en-1-one

It is also possible to observe a deviation in the signals of a few ppm. The deshielding in the doublet of the H3 proton when compared to the doublet of the starting material (unhydrolyzed compound) is quite clear (Figure 3.4). This deshielding could be due to the proximity of the oxygen heteroatom.

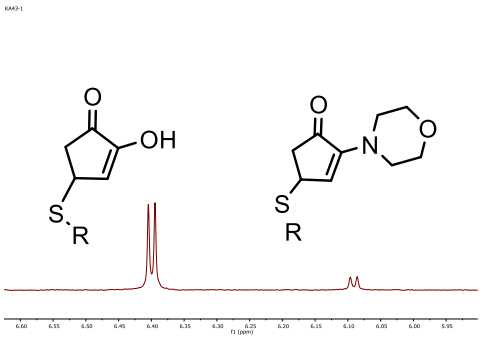
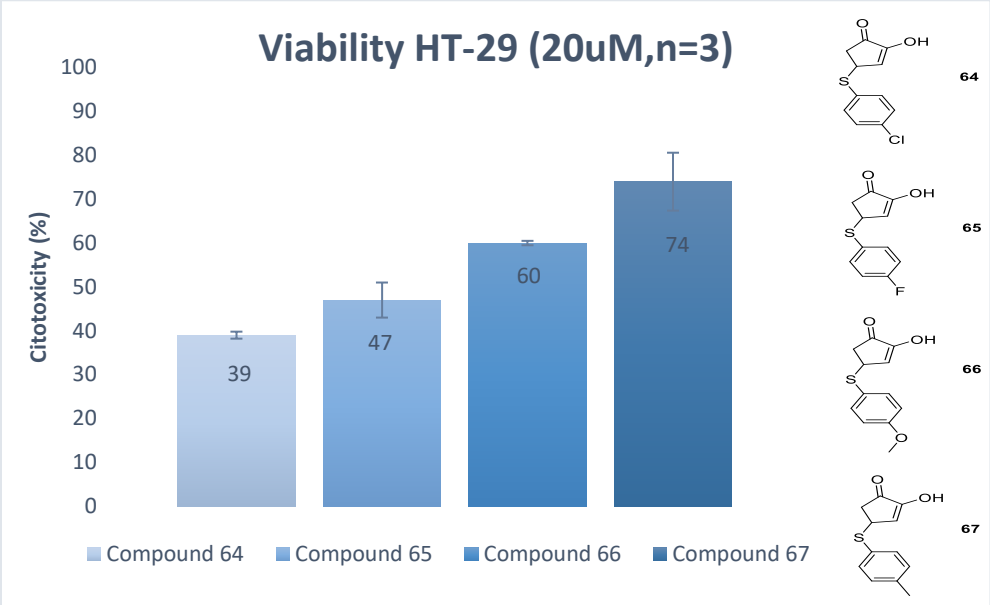


Figure 3.4 – Comparison of chemical shifts in NMR spectra of same proton by two different compounds

Regarding the biological assays, the hydroxy cyclopentenones were only tested in HT-29 cell line (in triplicate in order to validate the results). A small percentage of the impurity present in the product was taken into account in the calculations and dilutions of compound.



Graph 3.5 - Values of cellular viability obtained after exposure of the HT-29 colon tumour cells to 2-hydroxy-4-substituted-CPs at 20 µM, for a period of 48 hours. Viability was determined with the Neutral Red reagent and the experimental points represented are the mean of 3 replicates per experimental condition and the error experimental is the standard deviation

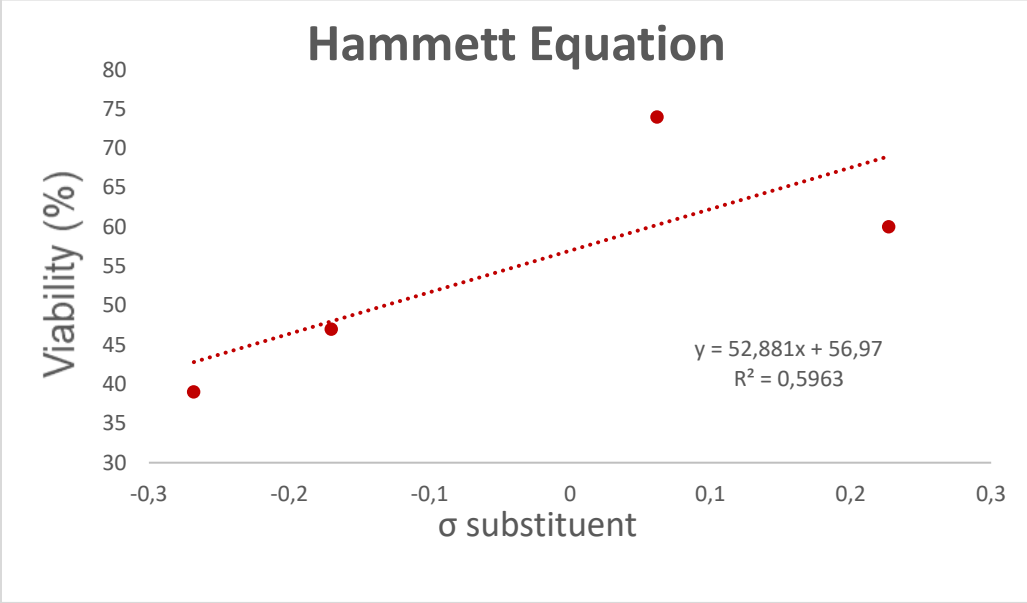
The standard deviation values of the hydroxy cyclopentenones are small, indicating that there is no significant discrepancy among the triplicates.

It is observed that all tested compounds contain the aromatic ring attached for the thiol, which due to its planarity must have some influence on the interactions with several targets and maybe on the cytotoxicity, but we cannot yet draw conclusions about its presence.

Interestingly, the cytotoxicity of the compounds increases with the electronic affinity of the phenol substituents. Electron affinity is related to the amount of energy released by an atom upon receiving an electron (hence the values are negative) and is influenced by electronegativity, atomic radius and ionization potential. Chlorine, for example, although it is less electronegative than fluorine, has a higher electro-affinity because fluorine has a small atomic radius and has a greater difficulty in accommodating the electron. Thus, the electron affinity varies like that:

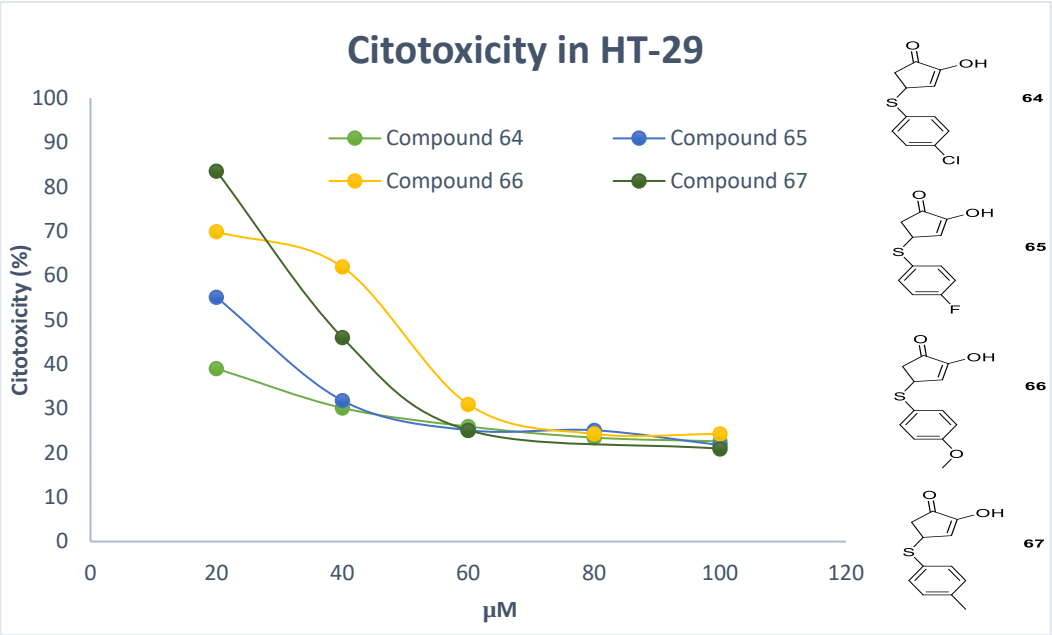
4-Cl > 4-F > 4-Me > 4-OMe

Thus, was possible to construct a linear correlation into the cellular viability values of each compound with the σ value of each substituent calculated by the Hammett equation based on the electron affinity. These results lead us to infer that if we carry out biological assays with substituents of the aromatic ring in position *para* with greater Electron affinity it is probable that we obtain better results of cytotoxicity (Graph 3.6).



Graph 3.6 – Linear correlation into the cellular viability values of 2-hydroxy-4-substituted-CPs at 20 µM for HT-29 colon tumour cells and σ value of each substituent

The previous results are relative to the concentration of 20µM, however assays were also performed at 40µM, 60µM, 80µM and 100µM. These values show an exponential decay as the concentration increases, as expected, however at high concentrations the values are quite similar, implying that from 100µM the viability of cells is almost null independent of the present substituent. So, 20µM appears to be a suitable concentration to measure the differences between toxicities for these compounds (Graph 3.7).

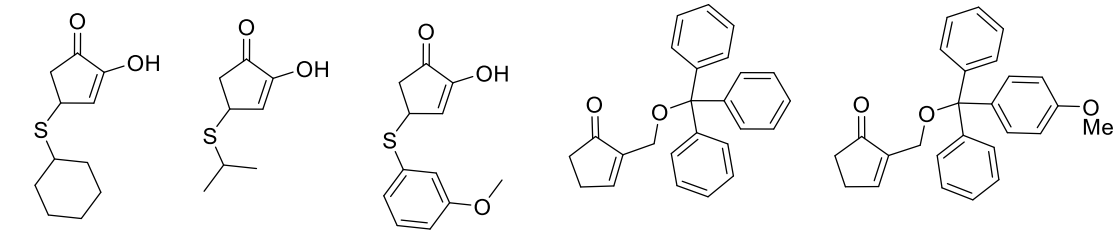


Graph 3.7 - Values of cellular viability obtained after exposure of the HT-29 colon tumour cells to 2-hydroxy-4-substituted-CPs at 20 µM, 40 µM, 60 µM, 80 µM and 100 µM for a period of 48 hours. Viability was determined with the Neutral Red reagent

The best way to compare the cytotoxicity of these compounds is by calculation theseIC_{50}, and for that we used the concentrations of 5µM, 10µM, 12µM; 15µM and 20µM and we resorted to the aid of the program GraphPad Prism 7.

With these results we can infer that the substituent at the C4 position would alter the cytotoxicity by a mechanism still unknown.

Our group has already prepared some CPs with other substituents at positions 2 and 4 and tested them on HT-29 (Scheme 3.9.).

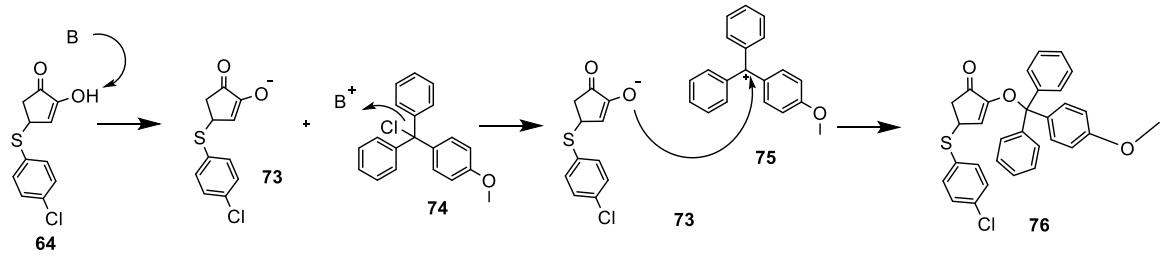


Scheme 3.9. – Compounds previously synthetized and their IC_{50}

From these previously prepared compounds we can observe that the hydroxy compound that showed the highest cytotoxicity (IC 50 = 13.7µM) is replaced in C4 by a methoxy-thiophenol which the methoxy is on meta position (**70**). Relatively to CP **66,** the methoxy is on para position, thus we need the IC_{50} value of our compound **66** in order to draw conclusions about the position of the phenol substituents.

CPs substituted by a bulk group in position 2 also exhibited considerable cytotoxic activity (**71** and **72**), however these compounds are good Michael acceptors and its activity may be due to such acceptor character. It was then thought to prepare a compound substituted with 4-chlorobenzenethiol at position 4 and chloro((4-methoxyphenyl) methylene) dibenzene at position 2 and to verify cytotoxicity to infer the role of the substituent in poor Michael acceptors CPs.

Initially, we attempted to protect the alcohol group of **64** by the same method as that was applied before for the synthesis of **72**. The mechanism of the reaction consists of two main steps: the addition of a base, in the case of pyridine, to deprotonate the alcohol, and also facilitate the formation of a carbocation; and protection. For this reaction we chose use the CP **64** that had best cytotoxic activity. (Scheme 3.10)



Scheme 3.10 – Proposed mechanism for 4-((4-chlorophenyl)thio)-2-((4-methoxyphenyl)diphenylmethoxy)cyclopent-2-en-1-one synthesis (Nucleophilic Substitution of fist order)

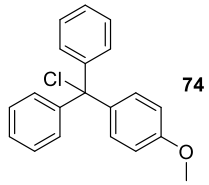
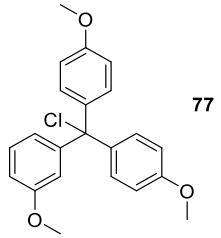
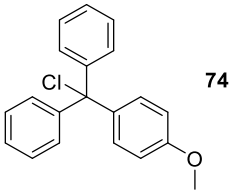
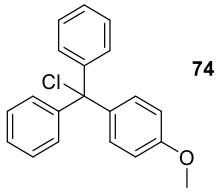
For the first attempt, TEA was used as the base and DMAP as a catalyst in dry DCM under dried conditions for approximately 24h. This reaction was repeated a few times and in all cases was observed the presence of **74** and by-products (Table 2).

We also tried to react with another protection group like 76 and it also did not work. We then exchanged the base for NaH, which is a stronger base, and the solvent for THF and allowed to react for 1 hour at 0 ° C and TLC and ^1H NMR observations showed degradation. When the base abstracts the proton from alcohol, various reactions can occur (Table 2).

Next, we used pyridine which is a weaker base with $AgNO_3$ to accelerate the reaction favouring the formation of the carbocation and once again the formation of many by-products was observed but in one of the fractions purified by chromatographic column it was possible to observe

the expected product, however the yield of the reaction was <5% and when we repeated it on a larger scale, we could no longer obtain it. We conclude that these conditions are also not adequate and that it is not worthwhile to continue to try to prepare this compound to draw conclusions about its activity (Table 2). Nevertheless, we can instead only methylate or acetylate the enol free hydroxyl group for possible modifications on the ketone group as reduction, formation of oxime and hydrazone formation in order to find out if the OH group and ketone group are involved on the mechanism of action.

Table 2 – Unsuccessful attempts for 4-((4-chlorophenyl)thio)-2-((4-methoxyphenyl)diphenylmethoxy)cyclopent-2-en-1-one synthesis

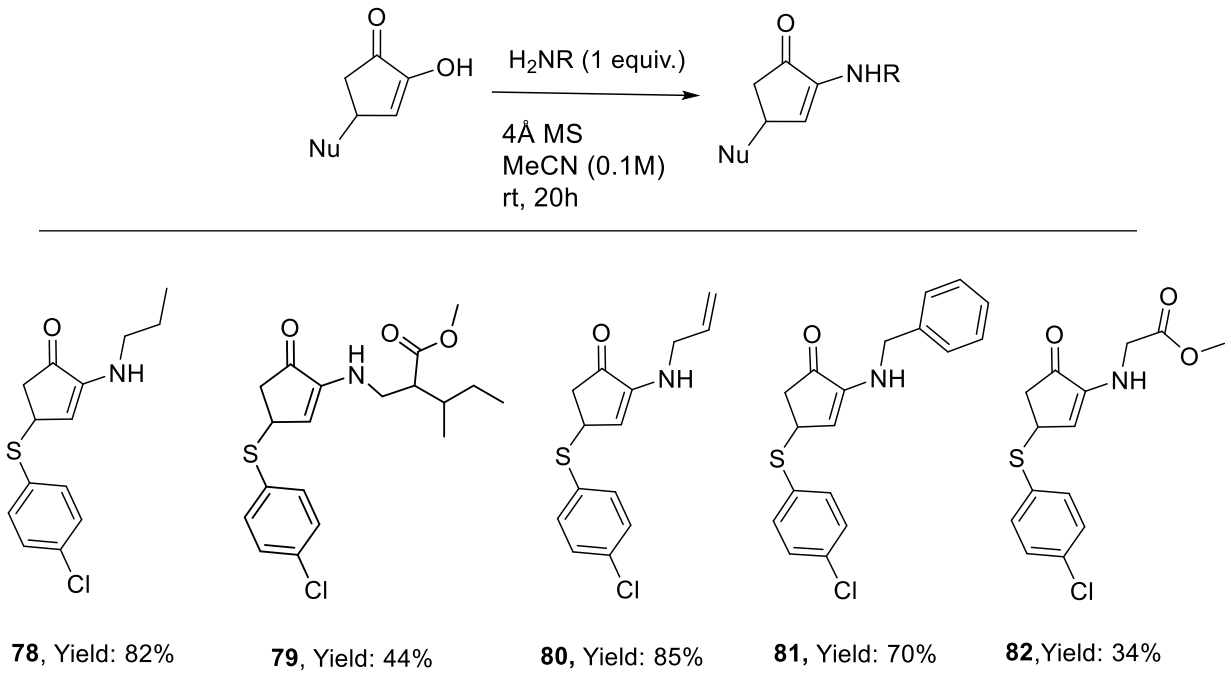
Protector	Solvent	Base	Promoter	Result
 **74**	DCM	TEA	DMAP	failure
 **77**	DCM	TEA	DMAP	failure
 **74**	THF	NaH	-	failure
 **74**	THF	Pyridine	$AgNO_3$	failure

The alcohol present on the CP used to synthetize CP **71** is a primary alcohol, while in **64** is a secondary alcohol (enol form) and the charge is not centred because of tautomerism. This difference leads us to infer that this is one of the reasons for the failure of reactions because the alcohol of 64 is more hindered than the alcohol of the previous one. The fact that **74** is a bulk compound makes it harder to react and this may be another reason for the failure. We also thought that other reason

could be due to the instability of the formed enol ether due to the ease of formation of a high stable carbanion.

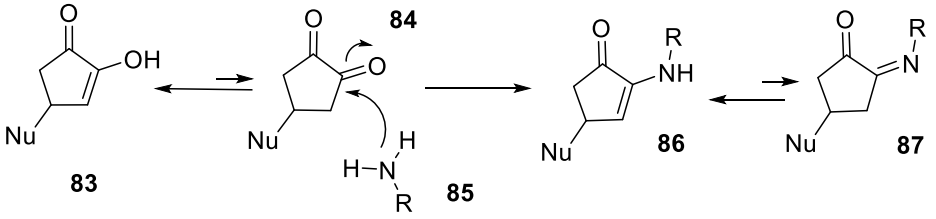
To continue our study on the importance of the substituent in position 2 we prepare CPs with secondary amines in the corresponding position. These amines retain the H-bond donor character of the free alcohol and allowed the addition of alkyl chains by potentiating Van der der Waals interactions with unknown targets.

The new CPs were prepared by the reaction of 4-((4-chlorophenyl)thio)-2-hydroxycyclopent-2-en-1-one (compound **64**) with primary amines including propan-1-amine, pentan-1-amine, prop-2-en-1-amine, phenylmethanamine and methyl glycinate acetonitrile following a proposed method. (Scheme 3.11).



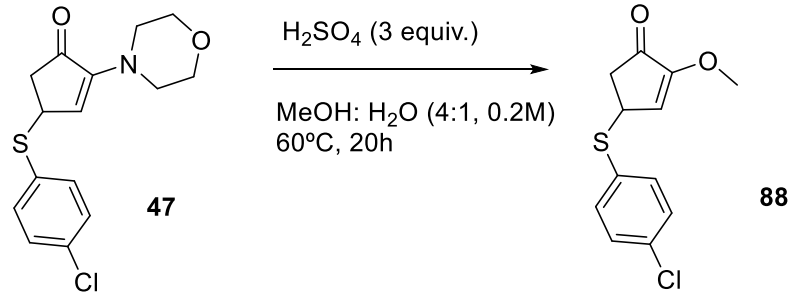
Scheme 3.11 – Above, the generic scheme for the synthesis of amine CPs from 2-hydroxy-4-substituted CPs in MeCN under acidic conditions

In the mechanism proposed the primary amine attacks the keto tautomeric form of **64** occurring elimination of water and formation of the enamine which is the most stable form (Scheme 3.12). In the NMR spectrum show all the same peaks of the **64** spectrum with exception of the peak from OH and also appears the peak from NH and the specifics peaks of each used amines.



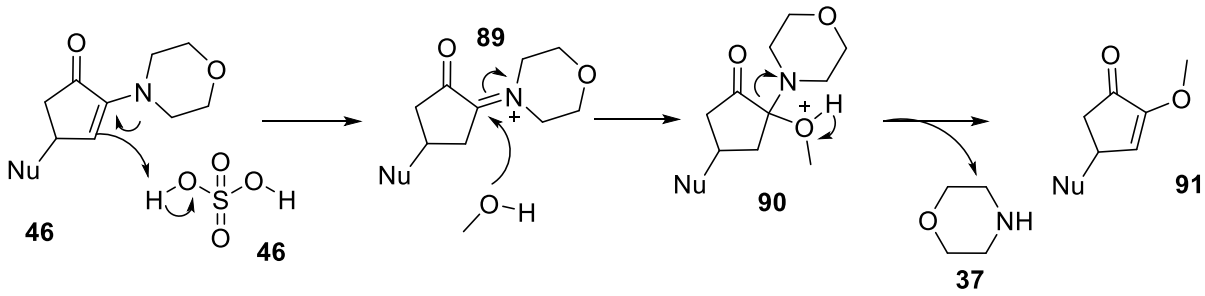
Scheme 3.12. – Proposed mechanism for amines CPs synthesis CPs from 2-hydroxy-4-substituted CP

In order to elucidate the importance of the alcohol group at position 2 for the mechanism of action of this type of compounds we reacted **47** by methanolysis in a mechanism similar to that of the previous hydrolysis to form the ester **88**. The reaction in this case was catalysed with sulfuric acid (3 equivalents) and the reaction time of 20h compared to the 2 hours of the hydrolysis (Scheme 3.13).



Scheme 3.13. – The scheme for the synthesis of 4-((4-chlorophenyl)thio)-2-morpholinocyclopent-2-en-1-one

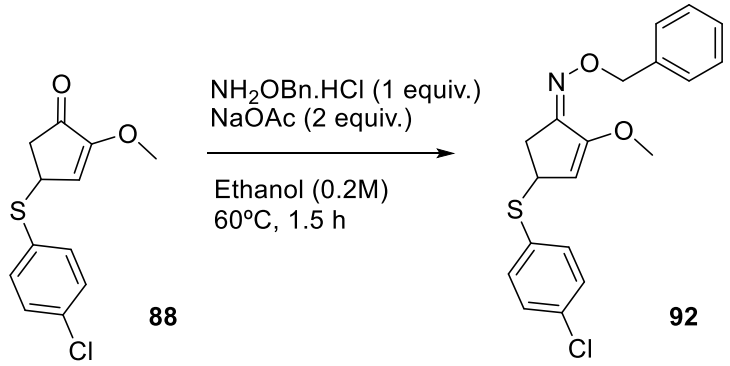
In this case, the enamine picks up a proton from the acid, and the free electrons of the amine delocalize affording an iminium cyclopentenone **89**, which can be attacked by methanol, eliminating the morpholine and forming the ester **91** (Scheme 3.14). The NMR spectra now shows the singlet of the methyl group.



Scheme 3.14. – Proposed mechanism for 2-methoxy-4-substituted CP's synthesis

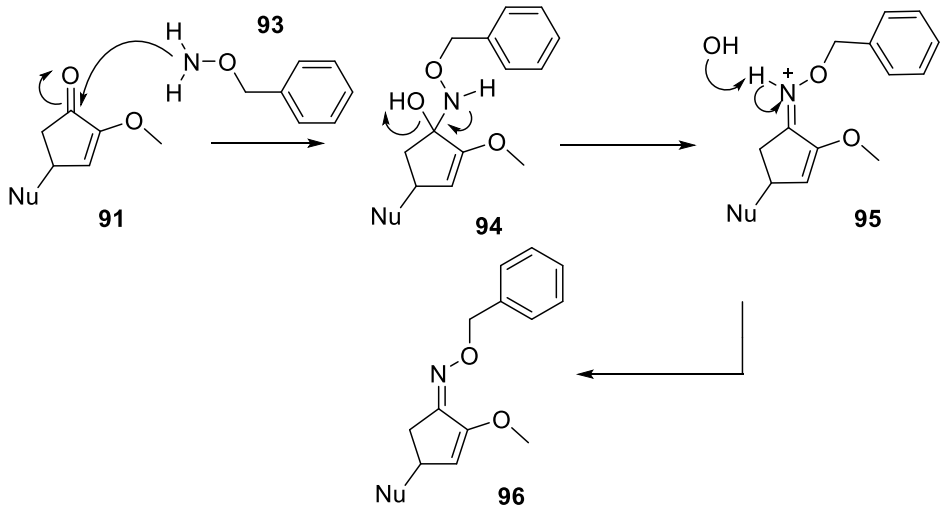
Finally, in order to verify the importance of the enone for the cytotoxicity we realize an oxime formation reaction that destroys the α, β unsaturated system. For that was used O-benzylhydroxylamine hydrochloride and a base to react with the acid in ethanol with temperature.

In oxime formation reactions 2 stereoisomers may appear, but we could only obtain one (Scheme 3.15).



Scheme 3.15. – The generic scheme for the synthesis of (Z)-4-((4-chlorophenyl)thio)-2-methoxycyclopent-2-en-1-one O-benzyl oxime

In the proposed mechanism O-benzylhydroxylamine that behaves as a nucleophile is added to the carbonyl group, and consequent proton transfers lead to the elimination of water forming the oxime (Scheme 3.16).



Scheme 3.16. – Proposed mechanism for oxime formation

The products of these last reactions with primary amines, the metanalysis and the formation of oxime haven't yet cytotoxic results.

3.2. Stability Assays

In order to prove the poor Michael Character of our compounds we decided to make stability studies of their under biological conditions in the presence of Glutathione. The Michael acceptors could be alkylated by Glutathione and simultaneously by other macromolecules critical to cells life. Thus, we were expecting the formation of an adduct into *trans*-4,5 amino-cyclopentenones and Glutathione by Michael Addiction and the non-formation of adducts by Michael Addiction in the remaining CPs.

Initially we attempted to perform our assays by High Performance Liquid Chromatography (HPLC), however this method proved to be inefficient when initially, in order to optimise the conditions, we tested the **64** in acetonitrile and appeared on the chromatogram more peaks beyond the peak of our compound. Our compound may not be stable in the chromatographic column and this may be the reason for the failure.

Therefore, we chose to perform the assays by NMR stability studies. For such purpose, we diluted our compounds in 2 types of solutions:

- Plasma solution (20%) + deuterated DMSO (80%)
- Glutathione solution (20%) + deuterated DMSO (80%)

In the plasma solution was used Human Plasma diluted in deuterated buffer and in GSH solution was used GSH diluted in deuterated buffer with pH adjustment to simulate physiological conditions. We used the percentage of 80% of deuterated DMSO to dilute the compounds in both solutions because these are more soluble in DMSO. Thus, in order to check the percentage of adduct formation, in the NMR tubes we used an internal standard (sodium acetate) probe.

The first compound analysed was the *trans*-4,5-morpholine cyclopentenone (**42**). This spectra in plasma solution at 5 minutes showed all the peaks related to the compound with special attention to the duplets at 6.35 ppm and 7.9 ppm referring to the protons of the double bond of α, β-unsaturated carbonyl group (figure 3.5)

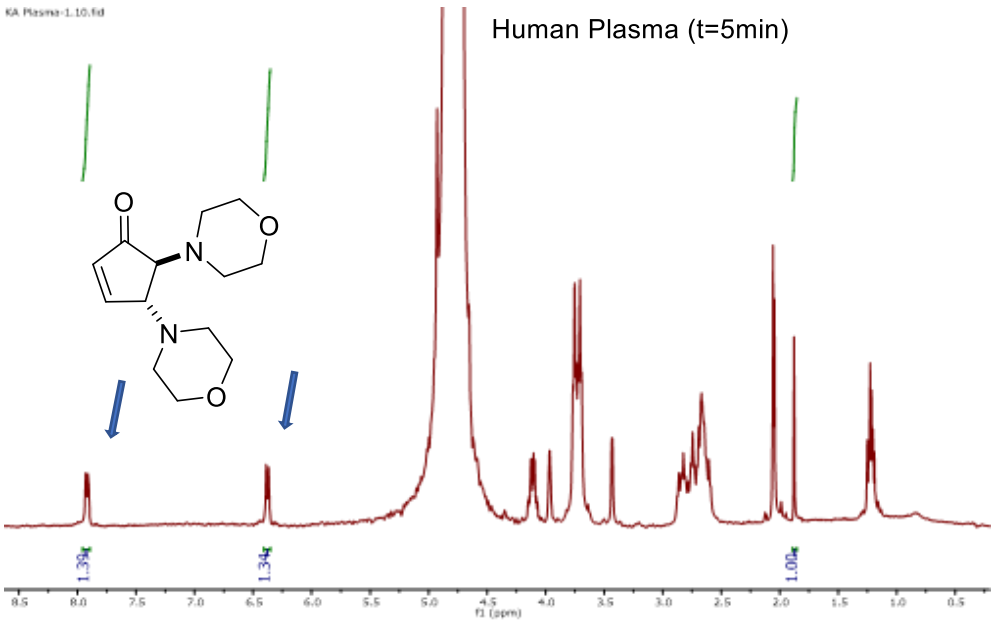


Figure 3.5. – NMR spectrum of (4R,5S)-4,5-dimorpholinocyclopent-2-en-1-one in Plasma Solution

However, the duplets related to the protons of the double bond of α, β-unsaturated carbonyl group do not appear in spectra of **42** in GSH solution at 5 minutes showed. This means that the electrophilic centre of this group was attacked by the sulfhydryl group of GSH by Michael Addition, but there is no evidence of the elimination of one morpholine (figure 3.6).

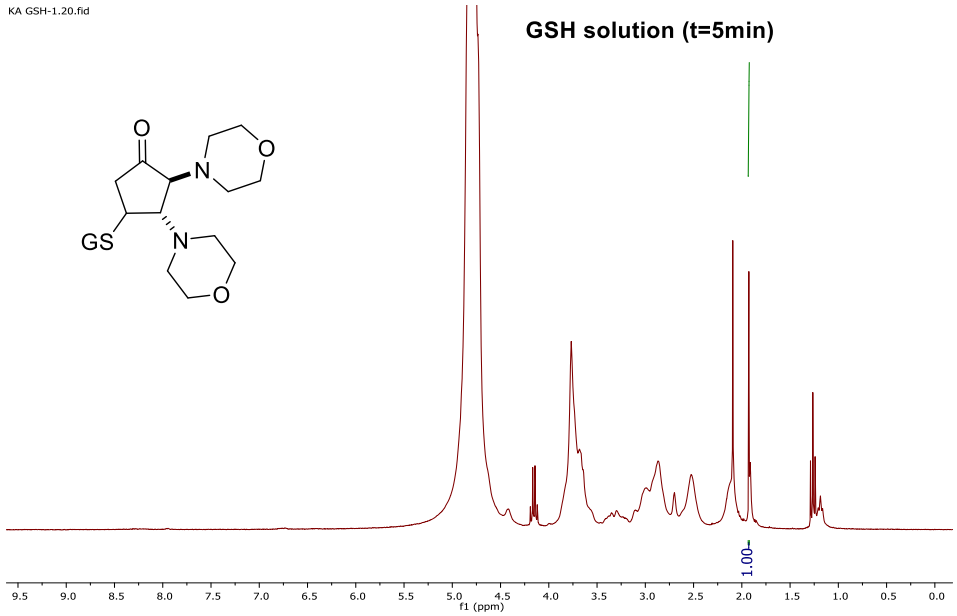


Figure 3.6. – NMR spectrum of (4R,5S)-4,5-dimorpholinocyclopent-2-en-1-one in GSH Solution after 5 minutes: formation of an intermediate

Thus, we have also analysed the same solution of GSH during 20h and the spectrum showed a presence of one new duplet at 6,65 ppm related to the proton of C3 which appears when one morpholine is eliminated. (figure 3.7)

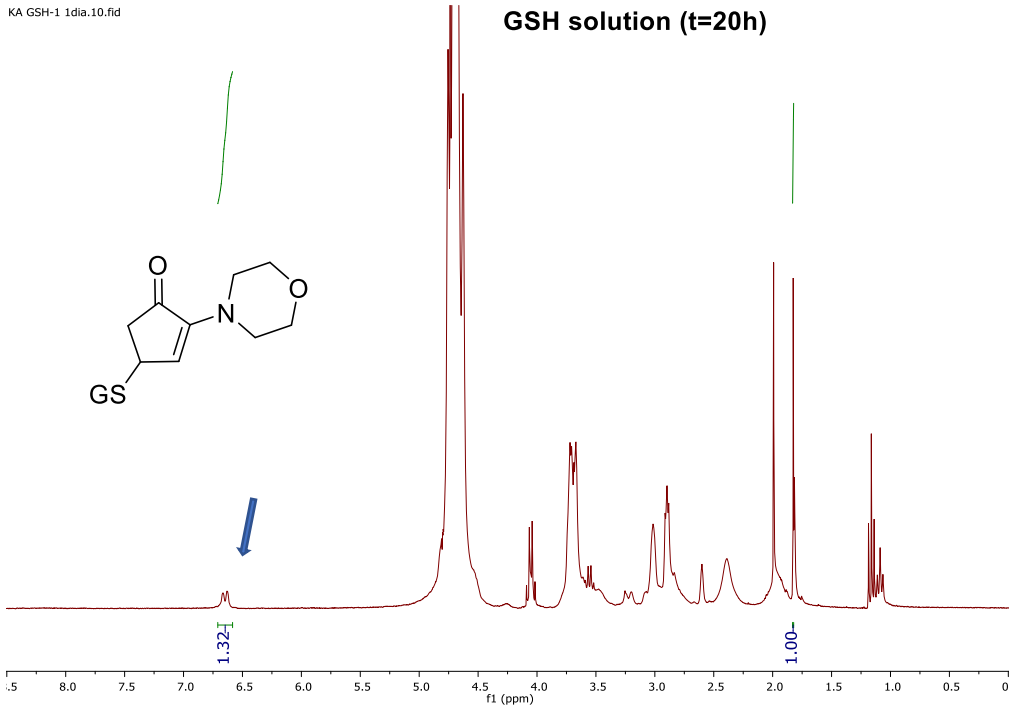
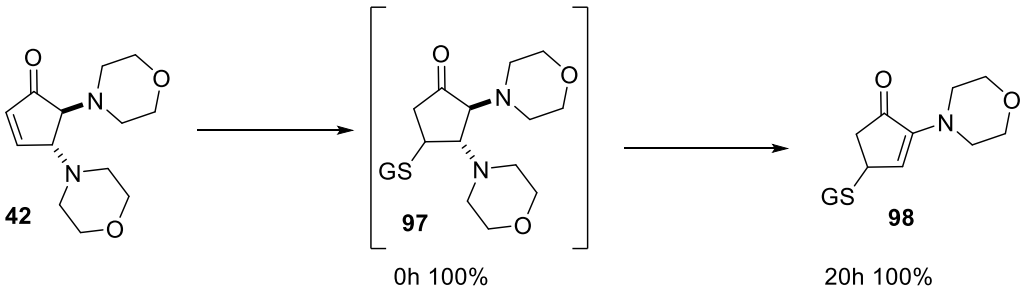
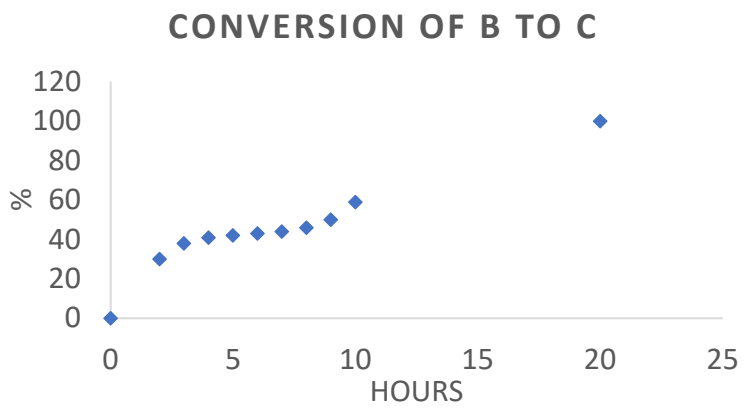


Figure 3.7. - NMR spectrum of (4R,5S)-4,5-dimorpholinocyclopent-2-en-1-one in GSH Solution after 5 minutes: formation of an adduct with GSH by Michael Addition

Thus, we can conclude that the compound 42 is a very good Michael Acceptor because it readily can form adducts with GSH by conjugated addiction, forming an intermediate **97** with 2 morpholines that and after some time the product **98** (Scheme 3.17).



Scheme 3.17. - General Scheme of formation of an adduct with GSH by Michael Addition



Graph 3.8. - Conversion of **97** to **98** during 20 h of reaction

We have also analysed the 4-((4-chlorophenyl)thio)-2-morpholinocyclopent-2-en-1-one (compound **47**). The spectra of it in Plasma solution showed all the peaks related to the compound with special attention for the duplet at 6,3 ppm related to the proton of the C3 and the multiplet at 7,4 ppm related to the four aromatic protons of the phenyl group present on the thiol.

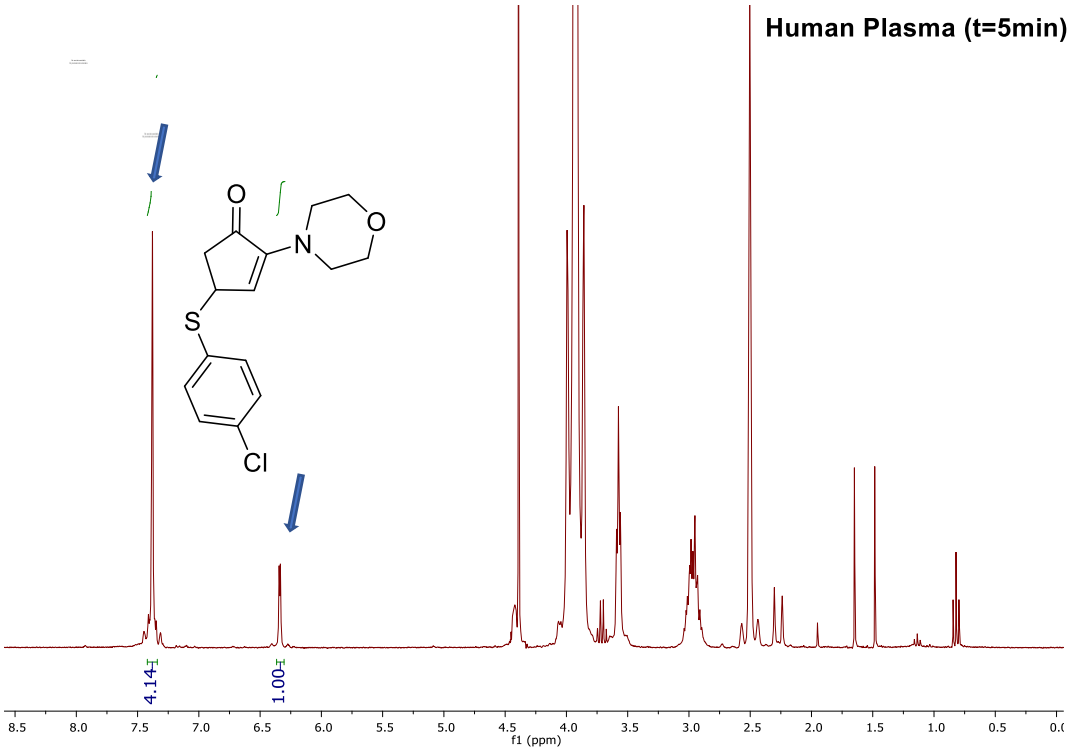


Figure 3.8. - NMR spectrum of 4-((4-chlorophenyl)thio)-2-morpholinocyclopent-2-en-1-one in Plasma Solution

The spectra of **47** in GSH solution at 5 minutes showed all the peaks related to the compound but at lower intensity, and new peaks appear relative to the aromatic protons, but this time they are not superimposed, which indicates that there was some reaction with GSH that interferes in the chemical deviation of the same ones and also appears a new relative peak to the carbon 3 proton of

this new compound. This means that there was no addition of Michael from glutathione to enone as expected and then we infer the formation of a compound whose GSH reacts in the same way as the primary amines eliminating the morpholine.

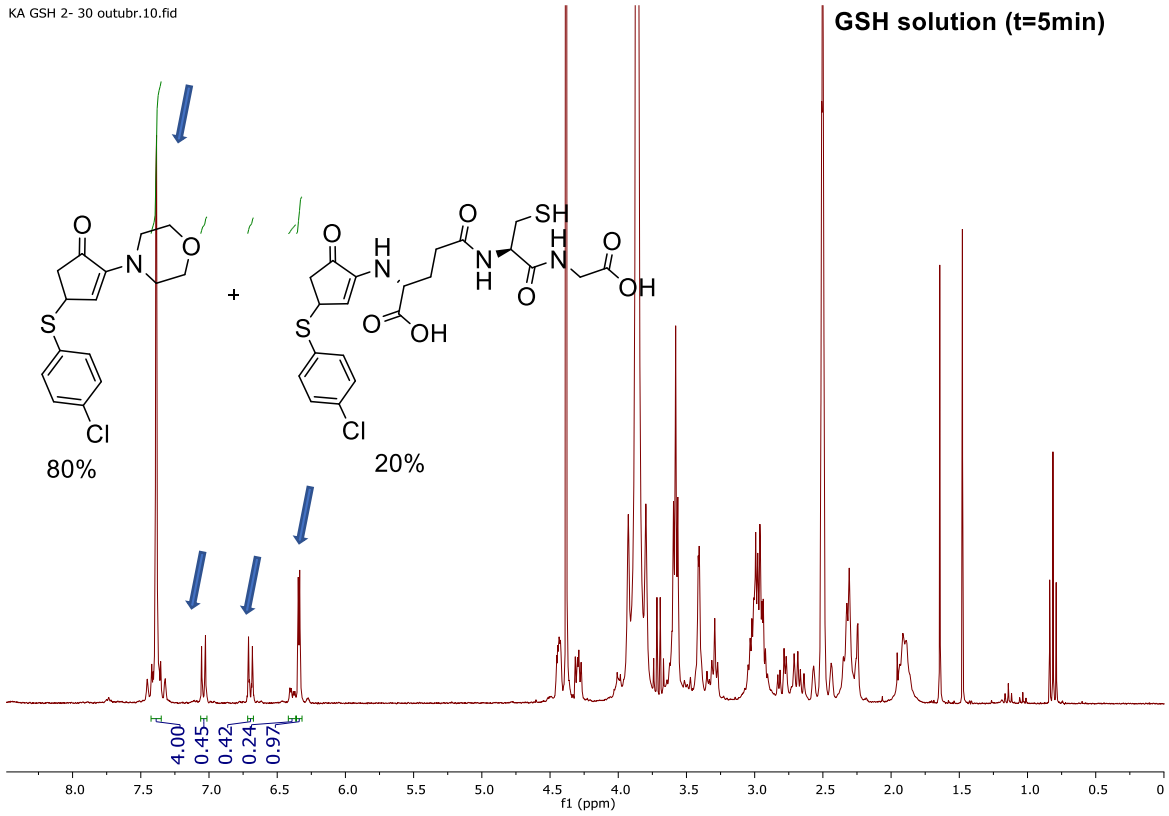
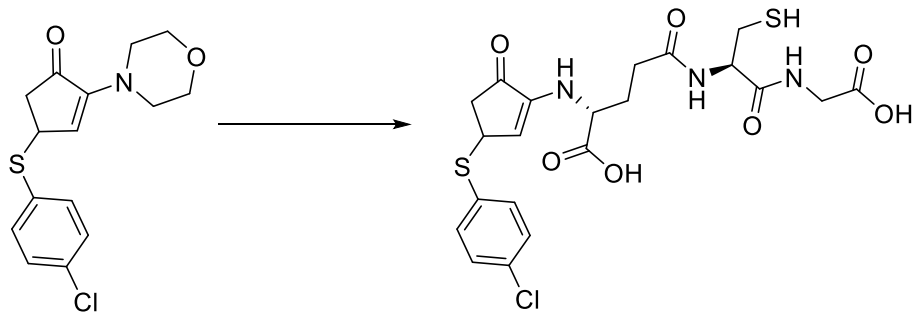


Figure 3.9. - NMR spectrum of 4-((4-chlorophenyl)thio)-2-morpholinocyclopent-2-en-1-one in GSH Solution after 5 minutes: 20% of formation of an adduct with GSH

Through the integral we verified the formation of 20% of this new compound. To confirm that this formed compound is actually the inferred B we will still carry out a reaction in Bath between A and GSH.



Scheme 3.18- General scheme for the formation of N5-((R)-1-((carboxymethyl)amino)-3-mercapto-1-oxopropan-2-yl)-N2-(3-((4-chlorophenyl)thio)-5-oxocyclopent-1-en-1-yl)-D-glutamine **99** from **47**

Afterwards, we analyzed the 4-((4-hlorophenyl)thio)-2-hydroxycyclopent-2-en-1-one (compound **64**) at the same conditions to the compound **47**. The spectra of it in Plasma solution showed all the peaks related to the compound with special attention for the duplet at 6,4 ppm related to the proton of the C3 and the multiplet at 7,4 ppm related to the four aromatic protons of the phenyl group present on the thiol.

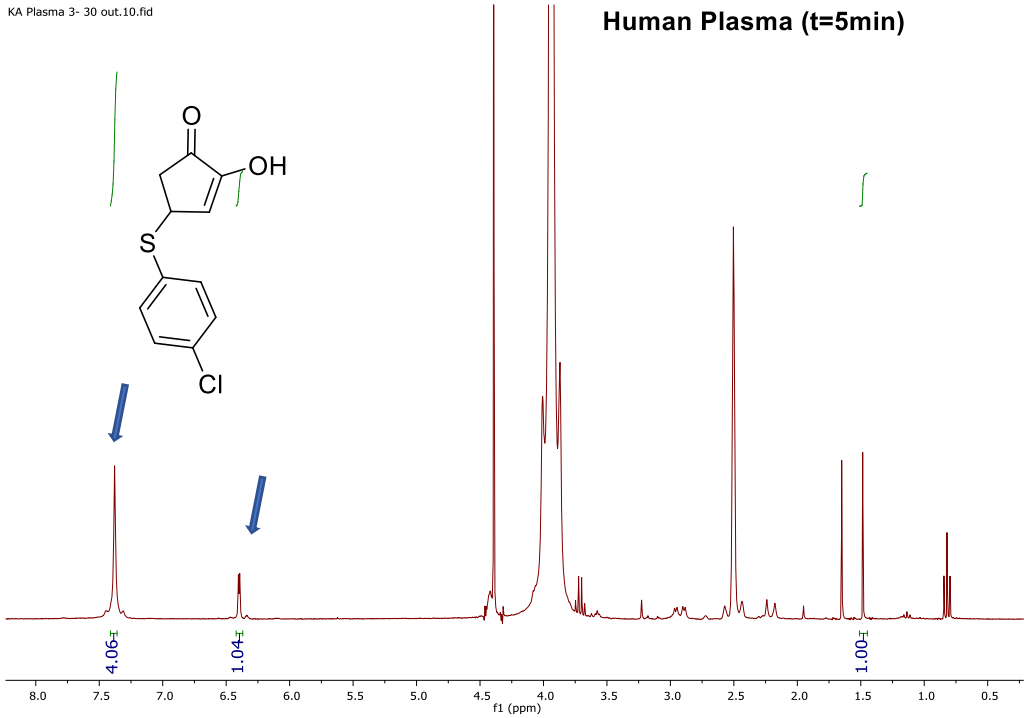


Figure 3.10. - NMR spectrum of 4-((4-hlorophenyl)thio)-2-hydroxycyclopent-2-en-1-one in Plasma Solution

The spectra of **47** in GSH solution at 5 minutes showed the same products that in the last case (peaks of **64** in worse intensity and new peaks of the aromatic protons and the proton of the double bond) but with a difference: the reaction with the hydrolysed compound is much faster consuming with about 80% of A in 5 minutes (Figure 3.11 and Scheme 3.9).

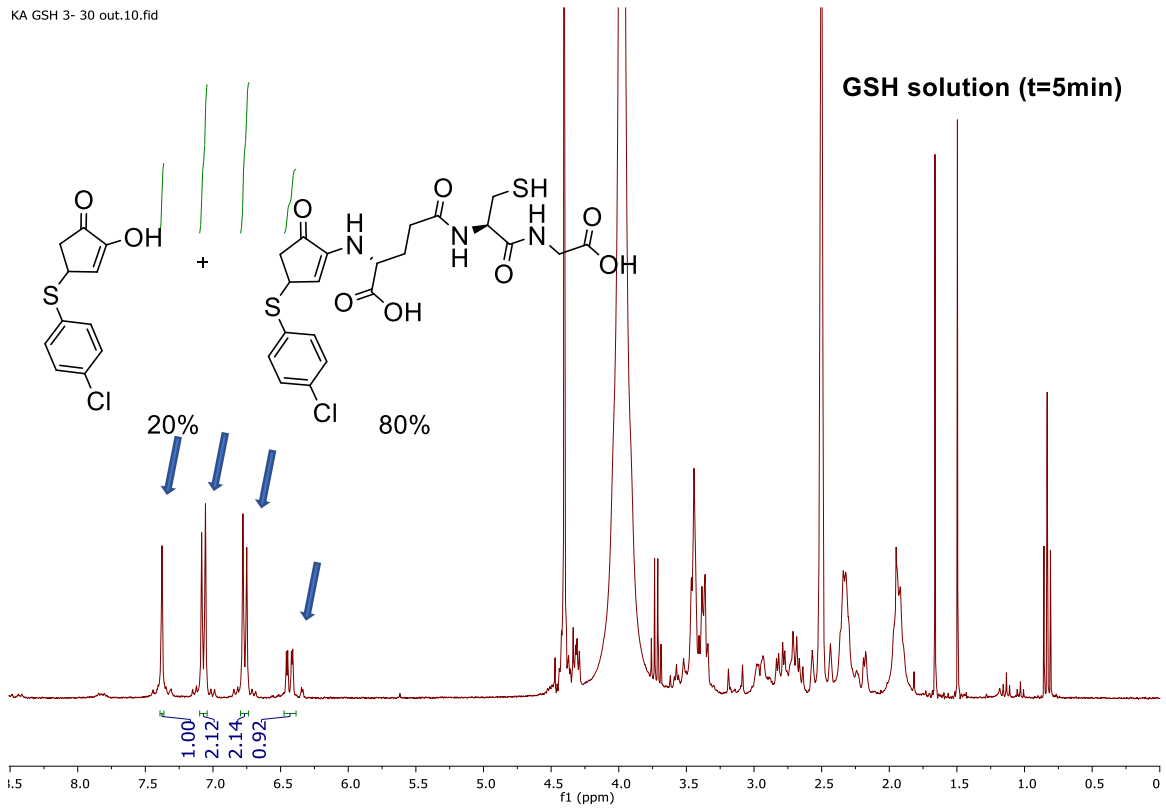
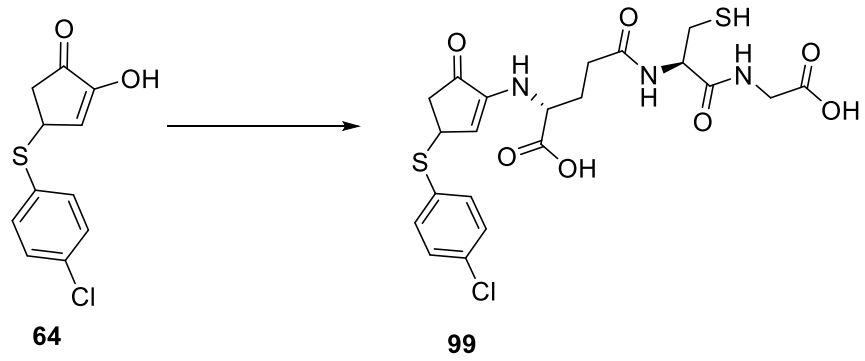


Figure 3.11. - NMR spectrum of 4-((4-hlorophenyl)thio)-2-hydroxycyclopent-2-en-1-one in GSH Solution after 5 minutes: 80% of formation of an adduct with GSH



64 **99**

Scheme 3.19. - General scheme for the formation of N5-((R)-1-((carboxymethyl)amino)-3-mercapto-1-oxopropan-2-yl)-N2-(3-((4-chlorophenyl)thio)-5-oxocyclopent-1-en-1-yl)-D-glutamine **99** from **64**

The results for these last compounds corroborate the idea that they are not good Michael Acceptors because morpholine present in **47** and the OH in **64** break the electrophilic character of C3 in the double bond of the of α, β-unsaturated carbonyl group.

4

Conclusions and Future Work

Our aim was to synthetize poor Michael acceptors with considerable cytotoxic activity against cancer cells to find a new cyclopentenones mechanism of action different to the known mechanisms by alkylation of the electrophilic centre of the enone.

We have synthetized a Michael acceptor precursor of these compounds, the 4,5-morpholine cyclopentenone, and evaluated its cytotoxicity against cancer cells and stability with GSH, and it showed no considerable cytotoxicity and as expected it formed adducts by Michael Addition with GSH.

We also have synthetized 2-morpholine-4-substituted cyclopentenones and have tested it with cancer cells and GSH, and these compounds showed some cytotoxicity (but not as significant), and no formation of adducts with Glutathione by Michael Addiction, it means that they are poor Michael acceptors. They also have good drug-like properties.

Finally, we have synthetized 2-hydroxy-4-substituted cyclopentenones and these compounds showed considerable cytotoxic activity against HT-29 colon tumour cancer cells. They were also tested with GSH and was observed no formation of adducts with Glutathione by Michael Addiction, it means that they are poor Michael acceptors. They also have good drug-like properties.

These results induce the hypothesis that there must be a mechanism of action still unknown other than the Michael addition that induces cell death.

For the determination of the structure structure activity we still need to test the novel compounds with substituent changes at the other enone positions. For future we also need to perform new biological essays to find the IC_{50} of all the synthetized compounds and perform new stability studies with glutathione in these to verify their promiscuity to form adducts by Michael addition.

5

Experimental Part

5.1 General Methods

Reagents and solvents:

All the reagents and solvents were obtained from commercial suppliers. The reagents were used without any purification with >98% purity (Sigma Aldrich, Alfa Aesar, Fluka), the solvents were distilled, and the anhydrous solvents prepared according usual procedures [132].

Stationary phases:

Silica gel 60A (P2050017, Carlo Erba) and (TA2045967, Merk) were used respectively for column chromatography and preparative TLC.

NMR spectroscopy:

^{1}H NMR spectra and ^{13}C NMR spectra were obtained in a Bruker Fourier 300 spectrometer. ^{1}H NMR spectra were recorded at 300 MHz and ^{13}C NMR were recorded at 100 MHz. The deuterated solvent used was $CDCl_3$ for normal spectrums and D_2O for stability assays. The chemical shifts are indicated in ppm, related to TMS, the *J* coupling constants in Hz, and the Splitting patterns are indicated as s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. Geminal protons are referred with “a” and “b” indexes.

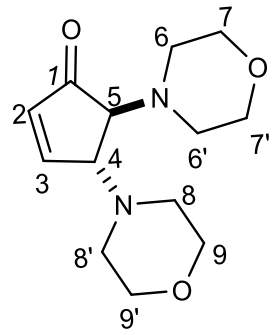
5.2 General procedure for *trans*-4,5-dimorpholinocyclopent-2-en-1-one synthesis

Where used two methods to synthetize *trans*-4,5-dimorpholinocyclopent-2-en-1-one. The quantities reported are just for one reaction, but was performed more than one reaction for each compound, and the yield is an average of yields for all the reactions

1) To a solution of furan-2-carbaldehyde (3g, 31.22mmol) in methanol (0.25M) was added 2 equivalents of morpholine, $MgSO_4$ (5g) and aluminium chloride (0.1eq). The mixture was stirred for six hours at room temperature under nitrogen atmosphere. The crude reaction was filtered with dichloromethane (DCM), brine was added, and the two layers were separated followed by extraction of the aqueous phase with DCM. Combined organic layers were dried ($MgSO_4$), filtered and concentrated under reduced pressure and the precipitated product (7.6g, 95%) was collected from the solution in satisfactory pure form.

2) To a solution of furan-2-carbaldehyde (3g, 31.22mmol) in acetonitrile (2M) was added 2 equivalents of morpholine and 0.1 equivalents of Erbium (III) chloride Hexahydrate. The mixture was stirred for 30 min at room temperature. The crude was filtered with dichloromethane (DCM), brine was added, and the two layers were separated followed by extraction the aqueous phase with DCM. Combined organic layers were dried ($MgSO_4$), filtered and concentrated under reduced pressure. Afterwards Diethyl Ether was added and the precipitated product (7.7g, 99%) was collected from the solution in satisfactory pure form.

Was afforded a pale yellow solid with an average yield of 97%. Spectral data in accordance to reported.[127]



1H NMR (300 MHz, $CDCl_3$): 2.63 (m, 6H), 2.82 (dt, *J* = 11.1 Hz, 4.4 Hz 2H), 3.29 (d, *J* = 3.1 Hz, 1H), 3.68 (t, *J* = 4.7 Hz, 4H), 3.72 (t, *J* = 4.6 Hz, 4H), 3.81 (m,1H), 6.23 (dd, *J* = 6.2, 2.2 Hz, 1H), 7.61 (dd, *J* = 6.2, 2.2 Hz, 1H);

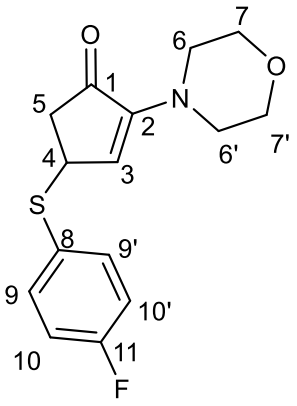
^{13}C NMR (100 MHz, $CDCl_3$): 49.95 (2C), 50.23 (2C), 66.73 (C5), 67.23 (2C), 67.41 (2C), 68.20 (C4), 135.56 (C3), 160.75 (C2), 206.21 (C1).

5.3 General procedure for morpholine cyclopentenone's synthesis

To a solution of *trans*-4,5-dimorpholinocyclopent-2-en-1-one in dried methanol (0.1M) was added 1 equivalent of the appropriate substituted thiol and 0.25 equivalents of potassium tert-butoxide. The mixture was stirred at room temperature under nitrogen atmosphere for 1-2h. Afterwards, the crude was filtered in a porous glass filter and celite with DCM, was added buffer (AcOH/NaOAc (pH=5), and brine and the two layers were separated followed by extraction of the aqueous phase with DCM (3 times). Combined organic layers were dried ($MgSO_4$), filtered and concentrated under reduced pressure.

The quantities reported are just for one reaction but was performed more than one reaction for each compound, and the yield is an average of yields for all the reactions.

5.3.1. 4-((4-fluorophenyl)thio)-2-morpholinocyclopent-2-en-1-one



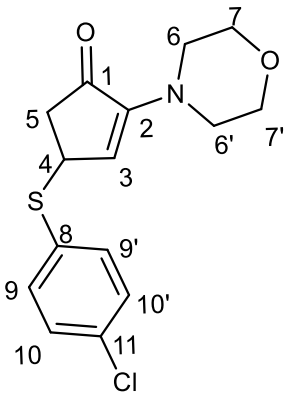
Following the general procedure, to a solution of *trans*-4,5-dimorpholinocyclopent-2-en-1-one (700 mg, 2.77mmol) in methanol was added 4-fluorobenzenethiol. The compound was purified by flash chromatography (EtOAc/*n*-hexane 1:9). Reaction time: 1h. Was afforded a light brown solid (43%)

1H-NMR (300 MHz, $CDCl_3$): 2.46 (dd, *J* = 19.2, 1.8 Hz, 1H, 5'H), 2.87 (dd, *J* = 19.2, 6.3 Hz, 1H, 5''H), 3.10 (*J* = 4.5, 2.4 Hz, 4H, H6, H6'), 3.75 (t, *J* = 4.8 Hz, 4H, H7, H7'), 4.21 (ddd, *J* = 6.3, 3.1, 1.8 Hz, 1H, H4), 6.15 (d, *J* = 3.1 Hz, 1H, H3), 7.03 (dd, *J* = 8.9, 8.5 Hz, 2H, H10, H10'), 7.41 (dd, *J* = 8.9, 5.3 Hz, 2H, H9, H9')

^{13}C NMR (100 MHz, $CDCl_3$) 43.46 (C5), 43.58 (C4), 47.94 (C6, C6'), 66.54 (C7, C7'), 116.24 (C10), 116.53 (C10'), 126.06 (C8), 130.62 (C3), 135.97 (C9'), 126.11 (C11), 136.06 (C9), 150.89 (C2), 201.99 (C1)

5.3.2. 4-((4-chlorophenyl)thio)-2-morpholinocyclopent-2-en-1-one

Following the general procedure, to a solution of *trans*-4,5-dimorpholinocyclopent-2-en-1-one (250 mg, 0.990 mmol) in methanol was added 4-chlorobenzenethiol. The compound was purified by flash chromatography (EtOAc/*n*-hexane 1:9). Reaction time: 1h. Was afforded a dark yellow solid (54 %).

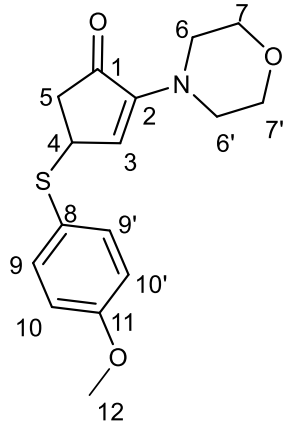


1H-NMR (300 MHz, $CDCl_3$): 2.43 (dd, *J* = 19.2, 1.7 Hz, 1H, H5'), 2.90 (dd, *J* = 19.2, 6.3 Hz, 1H, H5''), 3.11 (dt, *J* = 6.0, 4.7 Hz, 4H, H6, H6'), 3.74 (t, J= 4,8 Hz, 4H, H7, H7'), 4.27 (ddd, J = 6.3, 3.2, 1.8 Hz, 1H, H4), 6.15 (d, *J* = 3.1 Hz, 1H, H3), 7.39 -7.23 (m, 4H, H9, H9', H10, H10')

^{13}C NMR (100 MHz, $CDCl_3$) 43.00 (C5), 43.52 (C4), 47.86 (C6, C6'), 66.60 (C7, C7'), 129.36 (C10, C10'), 130.0 (C3), 132.11 (C8), 133.99 (C9, C9'), 134.12 (C11), 150.92 (C2), 201.75 (C1)

5.3.3. 4-((4-methoxyphenyl)thio)-2-morpholinocyclopent-2-en-1-one

Following the general procedure, to a solution of *trans*-4,5-dimorpholinocyclopent-2-en-1-one (800 mg, 3.17 mmol) in methanol was added 4-methoxybenzenethiol. The compound was purified by flash chromatography (EtOAc/*n*-hexane 1:9). Reaction time: 1h. Was afforded a brown solid. Spectral data compared to reported [128].

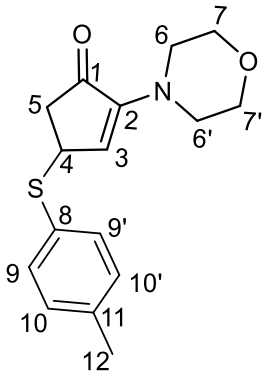


1H-NMR (300 MHz, $CDCl_3$): 2.44 (dd, J = 19.2, 1.7 Hz, 1H, H5'), 2.81 (dd, J = 19.2, 6.3 Hz, 1H, H5''), 3.07 (t, J = 3.8 Hz, 4H, H6, H6'), 3.72 (t, J = 3.8 Hz, 4H, H7, H7'), 3.78 (s, 3H, H12), 4.13 (ddd, J = 6.3, 3.1, 1.7 Hz, 1H, H4), 6.17 (d, J = 3.2 Hz, 1H, H3), 6.84 (m, 2H, H10, H10'), 7.97 (m, H9, H9')

^{13}C NMR (100 MHz, $CDCl_3$)

43.3 (C5), 43.8 (C4), 48.0 (C6, C6'), 55.5 (C12), 66.5 (C7, C7'), 114.7 (C10, C10'), 122.9 (C3), 131.7 (C8) 136.5 (C9, C9'), 150.7 (C2), 160.2 (C11), 202.3 (C1).

5.3.4. 2-morpholino-4-(p-tolylthio)cyclopent-2-en-1-one

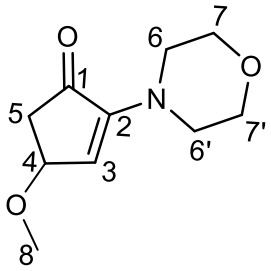


Following the general procedure, to a solution of *trans*-4,5-dimorpholinocyclopent-2-en-1-one (500 mg, 1.98 mmol) in methanol was added 4-methylbenzenethiol. The compound was purified by flash chromatography (EtOAc/*n*-hexane 1:9). Reaction time: 1h. Was afforded a dark brown solid (35%).

1H-NMR (300 MHz, $CDCl_3$): 2.23 (s, 3H, H12), 2.37 (dd, *J* = 19.2, 1.8 Hz, 1H, 5'H), 2.76 (dd, *J* = 19.2, 6.2 Hz, 1H, 5''H), 3.03 – 2.95 (m, 4H, H6, H6'), 3.67 – 3.59 (m, 4H, H7, H7'), 4.12 (ddd, J = 6.3, 3.2, 1.8 Hz, 1H, H4), 6.08 (d, *J* = 3.2 Hz, 1H, H3), 7.02 (d, J = 8.0 Hz, 2H, H10, H10'), 7.26 – 7.18 (d, J = 8.0 Hz, 2H, H9, H9')

^{13}C NMR (100 MHz, $CDCl_3$) 21.25 (C12), 43.13 (C5), 43.50 (C4), 47.93 (C6, C6'), 66.49 (C7, C7'), 129.43 (C8), 129.92 (C10, C10'), 131.25 (C3), 133.57 (C9, C9'), 138.29 (C11), 150.67 (C2), 202.19 (C1)

5.3.5. 4-methoxy-2-morpholinocyclopent-2-en-1-one



Following the general procedure, to a solution of 4,5-dimorpholinocyclopent-2-en-1-one (500 mg, 1.98 mmol) in methanol was added sodium methoxide. The compound was purified by flash chromatography (EtOAc/*n*-hexane 1:9). Reaction time: 1h. Was afforded a dark brown solid (30 and 35 %). Spectral data in accordance to reported.[127]

1H-NMR (300 MHz, $CDCl_3$): 2.33 (dd, J = 18.4, 1.8 Hz, 1H, H5'), 2.70 (dd, J = 18.3, 5.7 Hz, 1H, H5''), 3.15 (t, J = 4.9 Hz, 4H, H6, H6'), 3.36 (s, 3H, H8), 3.73 (t, J = 4.8 Hz, 4H, H7, H7'), 4.40 (ddd, J = 5.7, 3.0, 1.8 Hz, 1H, H3), 6.18 (d, J = 3.0 Hz, 1H, H4);

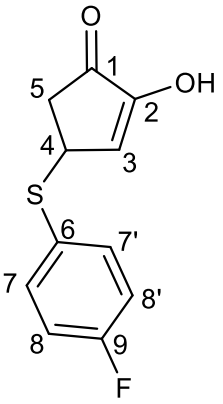
^{13}C NMR (100 MHz, $CDCl_3$): 42.5 (C5), 47.8 (C6, C6'), 56.5 (C8), 66.5 (C7, C7'), 74.4 (C4), 128.0 (C3), 151.4 (C2), 201.2 (C1).

5.4 General procedure for hydroxy cyclopentenone's synthesis

To a solution of the selected 2-morpholino-4-thio cyclopentenone **1** (0.2 M) in a mixture of MeOH/water (4:1 v/v) was added HCl 37% (1.1 eq.). The mixture was stirred at 60 ºC for 2 hours. Afterwards, water (30 mL) and DCM (20 mL) were added and the two layers were separated. The aqueous layer was further extracted with DCM (2 times of 15 mL). The combined organic layers were dried ($MgSO_4$), filtered and concentrated under reduced pressure to yield a solid which was purified by precipitation.

The quantities reported are just for one reaction, but was performed more than one reaction for each compound, and the yield is an average of yields for all the reactions

5.4.1 4-((4-fluorophenyl)thio)-2-hydroxycyclopent-2-en-1-one

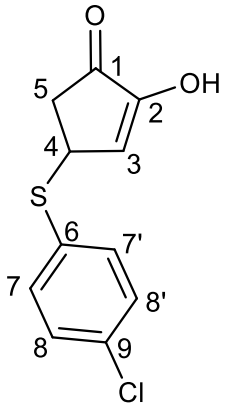


Following the general procedure, to a solution of 4-((4-fluorophenyl)thio)-2-morpholinocyclopent-2-en-1-one (40 mg, 0.136 mmol) in methanol was added chloride acid. The compound was purified by precipitation. Reaction time: 2h. Was afforded a brown solid (27%).

1H-NMR (300 MHz, $CDCl_3$): 2.44 (dd, *J* = 19.6, 1.5 Hz, 1H, H5'), 2.88 (dd, *J* = 19.6, 6.0 Hz, 1H), H5'', 4.22 (ddd, *J* = 6.0, 3.1, 1.5 Hz, 1H, H4), 6.46 (d, *J* = 3.0 Hz, 1H, H3), 7.08 – 6.98 (m, 2H, H7, H7'), 7.47 – 7.37 (m, 2H, H8, H8')

^{13}C NMR (100 MHz, $CDCl_3$): ^{13}C NMR (100 MHz, $CDCl_3$): 40.09 (C4), 42.25 (C5), 116.15 (C3), 116.44 (C8, C8'), 128.42 (C6), 136.31 (C7, C7'), 136.42 (C9), 153.13 (C2), 200.66 (C1)

5.4.2 4-((4-chlorophenyl)thio)-2-hydroxycyclopent-2-en-1-one

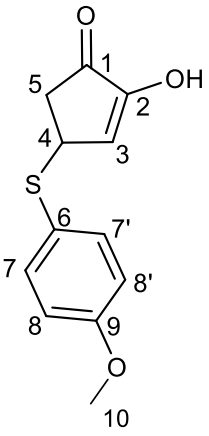


Following the general procedure, to a solution of 4-((4-chlorophenyl)thio)-2-morpholinocyclopent-2-en-1-one (3.5 g, 11.3 mmol) in methanol was added chloride acid. The compound was purified by precipitation. Reaction time: 2h. Was afforded a brown solid (25%).

1H-NMR (300 MHz, $CDCl_3$): 2.38 (dd, *J* = 19.6, 1.5 Hz, 1H, H5'), 2.85 (dd, *J* = 19.6, 6.0 Hz, 1H, H5''), 4.21 (ddd, J = 6.0, 3.1, 1.5 Hz, 1H, H4), 6.41 (d, *J* = 3.0 Hz, 1H, H3), 7.39 -7.23 (m, 5H, OH, H7, H7', H8, H8')

^{13}C NMR (100 MHz, $CDCl_3$): 40.52 (C5), 42.02 (C4), 128.67 (C3), 129.50 (C8, C8'), 131.17 (C6), 134.645 (C9), 134,67 (C7, C7') 153.67 (C2), 200.98 (C1)

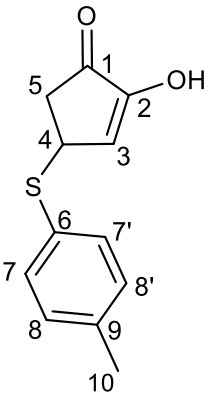
5.4.3 2-hydroxy-4-((4-methoxyphenyl)thio)cyclopent-2-en-1-one



Following the general procedure, to a solution of 4-((4-methoxyphenyl)thio)-2-morpholinocyclopent-2-en-1-one (100 mg, 0.327 mmol) in methanol was added chloride acid. The compound was purified by precipitation. Reaction time: 2h. Was afforded a dark brown solid (21%).

The spectra of H NMR and C NMR are not in this dissertation because it needs characterization and weren't yet possible.

5.4.4 2-hydroxy-4-(p-tolylthio)cyclopent-2-en-1-one



Following the general procedure, to a solution of 2-morpholino-4-(p-tolylthio)cyclopent-2-en-1-one (100 mg, 0.345 mmol) in methanol was added chloride acid. The compound was purified by precipitation. Reaction time: 2h. Was afforded a brown solid (24%).

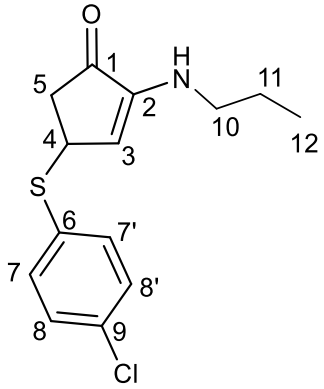
1H-NMR (300 MHz, $CDCl_3$): 2.34 (s, 3H, H10), 2.46 (dd, *J* = 19.7, 1.5 Hz, 1H, H5'), 2.88 (dd, *J* = 19.6, 5.9 Hz, 1H, H5''), 4.23 (ddd, J = 5.9, 3.0, 1.5 Hz, 1H, H4), 6.5 (d, *J* = 3.0 Hz, 1H, H3), 7.13 (d, *J* = 8.1 Hz, 2H), 7.33 (d, *J* = 8.1 Hz, 2H)

^{13}C NMR (100 MHz, $CDCl_3$) 21.30 (C10), 40.52 (C5), 42.12 (C4), 128.65 (C6), 129.60 (C3), 130.05 (C8, C8'), 138.72 (C9), 134.09 (C7, C7'), 153.36 (C2), 200.53 (C1)

5.5 General procedure for amine cyclopentenone's synthesis from hydroxy cyclopentenones

To a solution of the selected 4-((4-chlorophenyl)thio)-2-hydroxycyclopent-2-en-1-one in dried acetonitrile (0.1M) was added 1 equivalent of a specific primary amines and 4Å MS. The mixture was stirred at room temperature under nitrogen atmosphere for approximately 20h. Afterwards, water (30 mL) and DCM (20 mL) were added and the two layers were separated. The aqueous layer was further extracted with DCM (2 times of 15 mL). The combined organic layers were dried ($MgSO_4$), filtered and concentrated under reduced pressure to yield the product.

5.5.1 4-((4-chlorophenyl)thio)-2-(propylamino)cyclopent-2-en-1-one

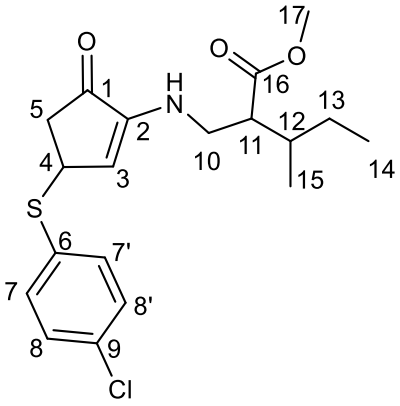


Following the general procedure, to a solution of 4-((4-chlorophenyl)thio)-2-hydroxycyclopent-2-en-1-one (50mg, 0.207mmol) in acetonitrile was added propan-1-amine. The compound was purified by flash chromatography (EtOAc/*n*-hexane 1:9). Was afforded a ligh brown oil (82 %).

1H-NMR (300 MHz, $CDCl_3$): 0.87 (t, *J* = 7.4 Hz, 3H, H12), 1.49 (h, 2H, H11), 2.39 (dd, *J* = 19.4, 1.6 Hz, 1H, H5), 2.93 – 2.75 (m, 3H, H10, H5), 4.28 (ddd, *J* = 6.0, 3.2, 1.6 Hz, 1H), 4.28 (ddd, *J* = 6.0, 3.2, 1.6 Hz, 1H, H4), 5.67 (d, *J* = 3.2 Hz, 1H, H3),

^{13}C NMR (100 MHz, $CDCl_3$) 11.63 (C12), 22.19 (C11), 44.40 (C5), 42.35 (C10), 45.99 (C4), 132.93 (C6), 117.58 (C3), 129.24 (C8, C8'), 133.70 (C9), 133.60 (C7, C7'), 146.95 (C2), 201.57 (C1)

5.5.2 4-((4-chlorophenyl)thio)-2-(pentylamino)cyclopent-2-en-1-one

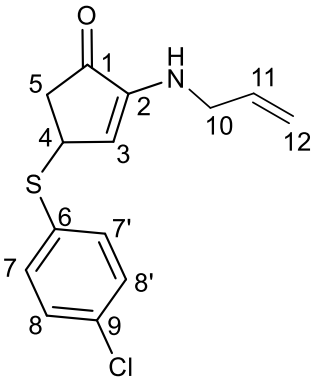


Following the general procedure, to a solution of 4-((4-chlorophenyl)thio)-2-hydroxycyclopent-2-en-1-one (50mg, 0.207mmol) in acetonitrile was added pentan-1-amine. The compound was purified by flash chromatography (EtOAc/*n*-hexane 1:9). Was afforded a dark brown oil (44 %).

1H-NMR (300 MHz, $CDCl_3$): 0.87 (dd, *J* = 9.7, 6.3 Hz, 6H, H14, H15), 1.70 – 1.46 (m, 4H, H11, H12, H13), 2.40 (dd, *J* = 19.5, 1.5 Hz, 1H, H5), 2.82 (dd, J = 19.5, 6.0 Hz, 1H, H5), 3.64 (s, 3H, H17), 3.73 (ddd, *J* = 8.6, 6.2 Hz, 1H, H4), 4.24 (ddd, *J* = 6.0, 3.1, 1.5 Hz, 2H, H10), 5.68 (d, *J* = 3.2 Hz, 1H, H3), 7.28 – 7.17 (m, 4H, H7, H7', H8, H8')

^{13}C NMR (100 MHz, $CDCl_3$) 21.94 (C14), 22.75 (C15), 24.78 (C13), 41.42 (C12), 42.19 (C4), 44.10 (C10), 52.23 (C5), 55.35 (C11), 119.25 (C17), 129.21 (C3), 132.20 (C8, C8'), 133.96 (C6, C9), 133.96 (C7, C7'), 145.75 (C2), 173.51 (C16), 200.83 (C1),

5.5.3 2-(allylamino)-4-((4-chlorophenyl)thio)cyclopent-2-en-1-one



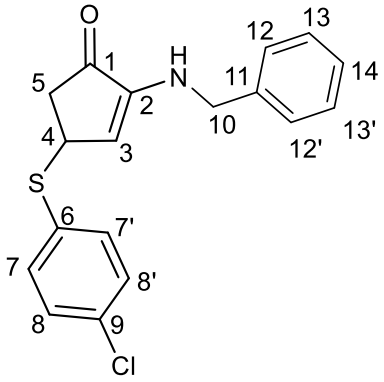
Following the general procedure, to a solution of 4-((4-chlorophenyl)thio)-2-hydroxycyclopent-2-en-1-one (50mg, 0.207mmol) in acetonitrile was added prop-2-en-1-amine. The compound was purified by flash chromatography (EtOAc/*n*-hexane 1:9). Was afforded a dark brown oil (85 %).

1H-NMR (300 MHz, $CDCl_3$): 2.38 (dd, *J* = 19.5, 1.5 Hz, 1H, H5), 2.81 (dd, *J* = 19.5, 6.0 Hz, 1H, H5), 3.56 (td, *J* = 5.3, 4.6, 2.6 Hz, 2H, H10), 4.12 (s,

1H, NH), 4.26 (ddd, *J* = 6.0, 3.1, 1.6 Hz, 1H, H4), 5.18 – 5.03 (m, 2H, H12), 5.81 – 5.63 (m, 2H, H3, H11), 7.29 – 7.17 (m, 4H, H7, H7', H8, H8')

^{13}C NMR (100 MHz, $CDCl_3$): 42.38 (C5), 44.30 (C10), 46.63 (C4), 116.69 (C12), 118.86 (C3), 129.21 (C6, C9), 132.61 (C11), 133.80 (C7, C7', C8, C8'), 146.58 (C2), 201.40 (C1).

5.5.4 2-(benzylamino)-4-((4-chlorophenyl)thio)cyclopent-2-en-1-one

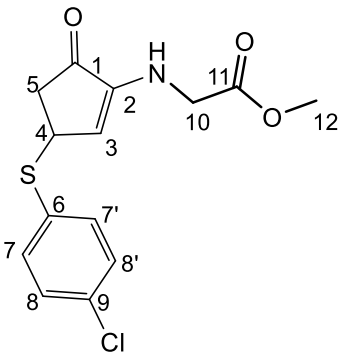


Following the general procedure, to a solution of 4-((4- chlorophenyl)thio)-2-hydroxycyclopent-2-en-1-one (50mg, 0.207mmol) in acetonitrile was added phenylmethanamine. The compound was purified by flash chromatography (EtOAc/*n*-hexane 1:9). Was afforded a light brown oil (70%).

1H-NMR (300 MHz, $CDCl_3$): 2.37 (dt, *J* = 19.4, 2.0 Hz, 1H), 2.81 (dd, *J* = 19.5, 6.0 Hz, 1H), 4.17 – 4.07 (m, 2H), 4.25 – 4.17 (m, 1H), 4.47 – 4.36 (m, 1H), 5.66 (t, *J* = 2.7 Hz, 1H), 7.33 – 7.15 (m, 10H)

^{13}C NMR (100 MHz, $CDCl_3$) 42.09 (C5), 44.30 (C4), 48.36 (C10), 119.32 (C3), 127.47 (C7, C7'), 127.63 (C14), 128.78 (C13, C13'), 129.22 (C12, C12'), 132.21 (C11), 133.94 (C6), 134.15 (C8, C8'), 137.89 (C9), 146.61 (C2), 201.44 (C1)

5.5.5 methyl (3-((4-chlorophenyl)thio)-5-oxocyclopent-1-en-1-yl)glycinate

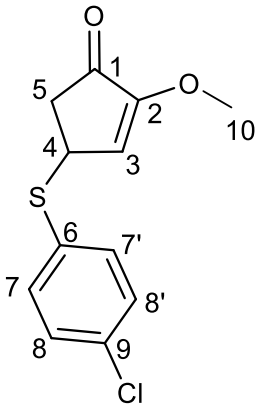


Following the general procedure, to a solution of 4-((4-chlorophenyl)thio)-2-hydroxycyclopent-2-en-1-one (50mg, 0.207mmol) in acetonitrile was added methyl glycinate. The compound was purified by flash chromatography (EtOAc/*n*-hexane 1:9. Was afforded a dark brown oil (34 %).

1H-NMR (300 MHz, $CDCl_3$): 2.40 (dd, *J* = 19.5, 1.6 Hz, 1H, H5), 2.91 – 2.78 (m, 1H, H5), 3.71 (s, 3H, H12), 3.72 (d, *J* = 1.8 Hz, 2H, H10), 4.27 (ddd, *J* = 6.1, 3.1, 1.6 Hz, 1H, H4), 5.70 (d, *J* = 3.1 Hz, 1H, H3), 7.29 – 7.21 (m, 4H, H7, H7', H8, H8')

^{13}C NMR (100 MHz, $CDCl_3$) 42.24 (C4), 44.10 (C10), 45.68 (C5), 52.51 (C12), 119.54 (C3), 129.37 (C8, C8'), 129.42 (C9), 132.58 (C6), 133.77 (C7, C7'), 146.20 (C2), 170.24 (C11), 200.65 (C1)

5.6 General procedure for 4-((4-chlorophenyl)thio)-2-methoxycyclopent-2-en-1-one's synthesis

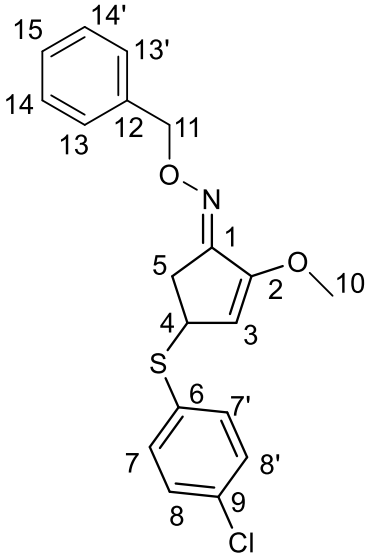


To a solution of 4-((4- chlorophenyl)thio)-2-hydroxycyclopent-2-en-1-one (1.2 g, 3.87 mmol) in a mixture of MeOH/water (4:1 v/v) was added H_2SO_4 (3 eq.). The mixture was stirred at 60 ºC for 20 hours. Afterwards, water (30 mL) and DCM (20 mL) were added and the aqueous layer was further extracted with DCM (30 mL). The combined organic layers were dried ($MgSO_4$), filtered and concentrated under reduced pressure.The compound was purified by flash chromatography (EtOAc/*n*-hexane 1:9). Was afforded a light brown solid (81 %).

1H-NMR (300 MHz, $CDCl_3$): 2.37 (dd, *J* = 19.5, 1.5 Hz, 1H, H5'), 2.83 (dd, *J* = 19.5, 6.2 Hz, 1H, H5''), 3.64 (s, 3H, H10), 4.22 (ddd, J = 6.6, 3.1, 1.7 Hz, 1H, H4), 6.21 (d, *J* = 2.9 Hz, 1H, H3), 7.33 -7.10 (m, 4H, H7, H7', H8, H8')

^{13}C NMR (100 MHz, $CDCl_3$): 40.41 (C5), 41.64 (C4), 57.47 (C10), 125.91 (C3), 129.26 (C8, C8'), 131.51 (C6), 133.95 (C7, C7'), 134,18 (C9) 158.15 (C2), 199.03 (C1)

5.7 General procedure for (E)-4-((4-chlorophenyl)thio)-2-methoxycyclopent-2-en-1-one O-benzyl oxime



To a solution of 4-((4-chlorophenyl)thio)-2-methoxycyclopent-2-en-1-one (50mg, 0.196 mmol) in ethanol (0.2M) was added O-benzylhydroxylamine (1 equiv.) and NaOAc (2 equiv.) The mixture was refluxed at 40ºC for 1,5 hours and afterwards washed with water (20mL) and extracted with DCM (20mL). The combined organic layers were dried ($MgSO_4$), filtered and concentrated under reduced pressure.The compound was purified by flash chromatography (EtOAc/*n*-hexane 1:9). Was afforded a dark brown oil (49 %).

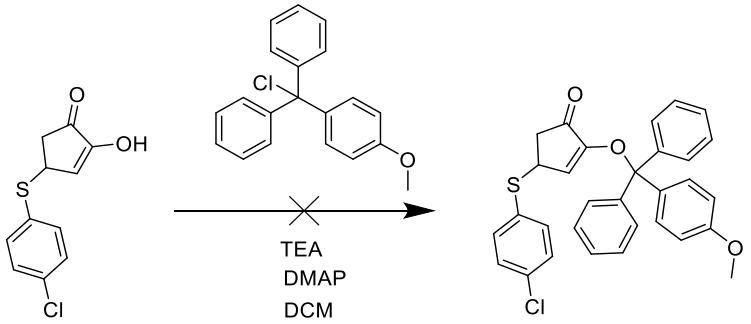
1H-NMR (300 MHz, $CDCl_3$): 2.71 (dd, *J* = 19.2, 1.7, 0.6 Hz, 1H, H5), 3.08 (dd, *J* = 19.2, 6.8 Hz, 1H, H5), 3.68 (s, 3H, H10), 4.19 (ddd, *J* = 6.8, 2.8, 1.7 Hz, 1H, H4), 5.09 (s, 2H, H11), 5.32 (d, *J* = 2.8 Hz, 1H, H3), 7.50 – 7.07 (m, 9H, H7, H7', H8, H8', H13, H13', H14, H14', H15)

^{13}C NMR (100 MHz, $CDCl_3$) 34.00 (C5), 45.49 (C4), 57.52 (C10), 76.83 (C11), 111.13 (C3), 128.03 (C15), 128.27 (C13, C13')128.49 (C14, C14'), 129.28 (C8, C8'), 132.99 (C6), 133.17 (C7, C7'), 133.56 (C9), 137.51 (C12), 156.15 (C2), 156.98 (C1)

5.8 General procedure for the attempts of 4-((4-chlorophenyl)thio)-2-((4-methoxyphenyl)diphenylmethoxy)cyclopent-2-en-1-one's synthesis

5.8.1 First Attempt:

To a solution of 4-((4-chlorophenyl)thio)-2-hydroxycyclopent-2-en-1-one (125 mg, 0.519 mmol) in dried dichloromethane (0.25M) was added 1.1 equivalents of Triethylamine, 0.02 equivalents of 4-Dimethylaminopyridine (DMAP) and 1.1 equivalents of chloro(4-methoxyphenyl)methylene)dibenzene in nitrogen atmosphere for 23h. The organic phase was washed with brine and DCM. The organic layer was dried over anhydrous $MgSO_4$ and concentrated at low pressure to yield a yellow oil. The TLCs and NMR spectra have showed several by-products.

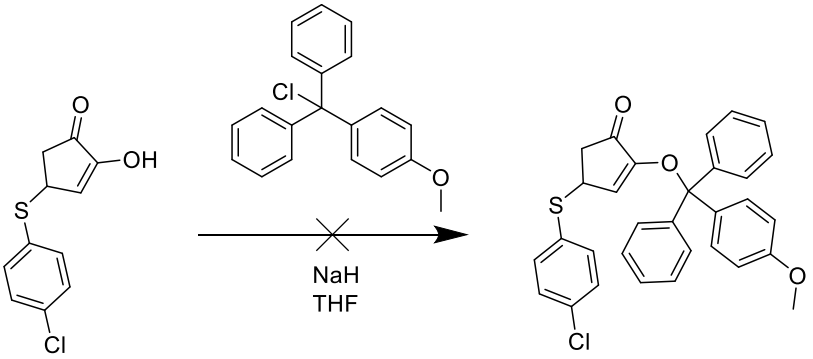


Scheme 5.1 – First attempt to the synthesis of 4-((4-chlorophenyl)thio)-2-((4-methoxyphenyl)diphenylmethoxy)cyclopent-2-en-1-one in DCM ,at basic conditions, catalyzed by DMAP

5.8.2 Second Attempt:

To a solution of 4-((4-chlorophenyl)thio)-2-hydroxycyclopent-2-en-1-one (125 mg, 0.519 mmol) in dried tetrahydrofuran (0.3M) was added 5 equivalents of sodium hydride (60%) and the mixture was stirred at 0ºC in nitrogen atmosphere for 1 hour. Afterwards was added 1.1 equivalents of chloro(4-methoxyphenyl)methylene)dibenzene and the mixture was stirred at R.T., overnight (14h). The organic phase was washed with brine and the mixture was extracted with DCM. The organic layer

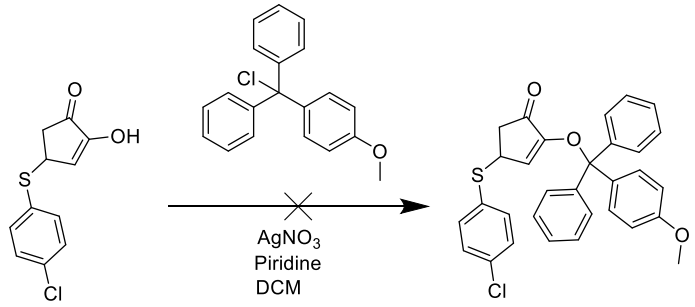
was dried over anhydrous $MgSO_4$ and concentrated at low pressure to yield a brown oil. The TLCs and NMR spectra have showed several by-products.



Scheme 5.2 – First attempt to the synthesis of 4-((4-chlorophenyl)thio)-2-((4-methoxyphenyl)diphenylmethoxy)cyclopent-2-en-1-one in THF ,at basic conditions.

5.8.3 Third Attempt:

To a solution of 4-((4-chlorophenyl)thio)-2-hydroxycyclopent-2-en-1-one in dried Dichloromethane (1M) was added 2 equivalents of pyridine, 1.5 equivalents of (chloro(4-methoxyphenyl)methylene)dibenzene and 2 equivalents of silver nitrate under nitrogen atmosphere at 0° C. The mixture was stirred at R.T., for 14h. After filtration, the organic phase was washed with brine and $NaHCO_3$ aqueous and the mixture was extracted with DCM. The organic layer was dried over anhydrous Na_2SO_4 and concentrated at low pressure to yield a yellow oil. The TLCs and NMR spectra have showed several by-products.



Scheme 5.3 – First attempt to the synthesis of 4-((4-chlorophenyl)thio)-2-((4-methoxyphenyl)diphenylmethoxy)cyclopent-2-en-1-one in DCM ,at basic conditions, catalyzed by $AgNO_3$

5.9 Cytotoxicity assays in human cell lines

Cytotoxicity assays were performed in breast, lung and colon cancer cell lines obtained from American Type Culture Collection HEK 293T – human embryonic kidney epithelial cell line using the Neutral Red method.

The breast cancer cell lines MCF-7 (ATCC HTB-22™), lung cancer cell lines NCI-H460 (ATCC HTB-177™) and colon cancer cells lines HT-29 (ATCC HTB-38™) are cryopreserved in a growth medium supplemented with 5% (v/v) DMSO at liquid nitrogen vapor phase and for the experiments are thawed and incubated in RPMI 1640 R8758, Sigma) culture medium supplemented with 10% fetal bovine serum (FBS) and antibiotic antimycotic solution (A5955 100x Sigma) at 37ºC in humidified 5% CO_2 atmosphere. The DMSO present are removed changing the medium.

To perform the assays the cells were counted by a Neubauer chamber cell count, diluted in RPMI 1640 culture medium supplemented with 10% fetal bovine serum, seeded in 96 well tissue culture plates (100µl per well) and incubated (37ºC, 5% CO_2 atmosphere). Concentrações de células utilizadas consoante a linha celular: 1x105 cel/ml (HT-29), 5x104 cel/ml (NCI-H460) e 1,5x105 cel/ml (MCF-7).

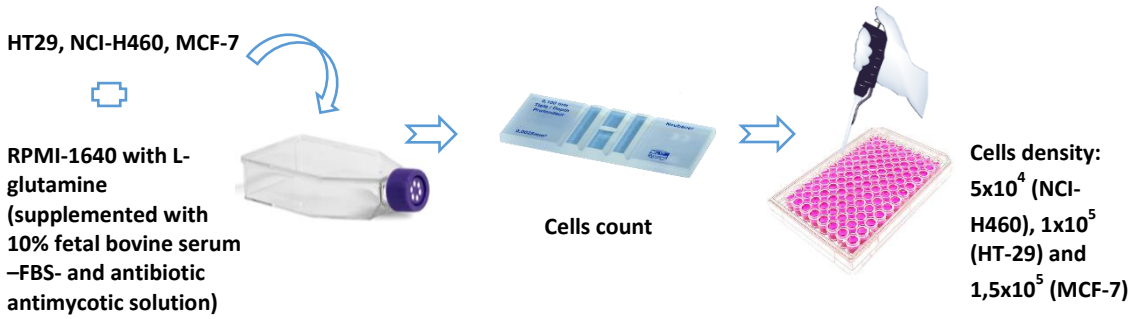


Figure 5.1 – Procedure of incubation, count and seeded of the cells

The compounds were dissolved in dimethyl sulfoxide (DMSO for cells culture) serially diluted in the culture medium (the final concentration of DMSO in culture medium during treatment did not exceed 0.5% (v/v)) and then, were added to the cells, previously seeded on the day before, and incubated for 48h. The same concentration of DMSO present in the wells with compounds was added to a well control, because the DMSO is also cytotoxic to cells.

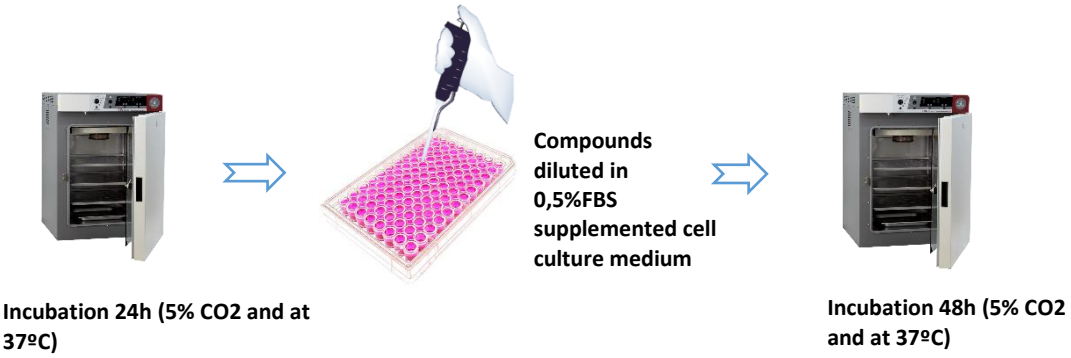


Figure 5.2 – Procedure of incubation of compounds with the cells

After 48 hours, for determine viability we used the Neutral Red reagent. We dilute the stock solution in culture medium to obtain the concentration of 50ug/ml in in contact with cells and incubate for 3 hours. After treatment with Neutral Red we remove the medium and wash the plate with PBS and then an organic solution (20 mls ethanol + 20mls H 2 O + 400 glacial acetic acid) is added and the absorbance at 540 nm is measured in the plate reader.

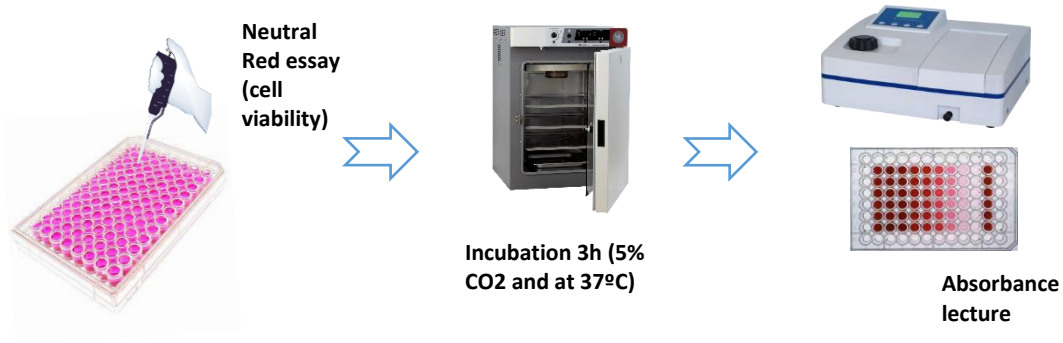


Figure 5.3 – Procedure to the determination of viability from Neutral Red method.

5.10 Stability assays with Glutathione

The stability assays were performed by NMR spectroscopy and the ^{1}H NMR spectra were obtained by DMSO deuterated experiences.

For all the experiences, was used an internal standard probe. The standard chosen was Sodium Acetate prepared in D2O (1 equiv. regarding to the quantity of compound).

Below are the conditions used to perform the assays to the following compounds (table x):

- *trans*-4,5-dimorpholinocyclopent-2-en-1-one (compound 42)
- 4-((4-chlorophenyl)thio)-2-morpholinocyclopent-2-en-1-one (compound 47)
- 4-((4-hlorophenyl)thio)-2-hydroxycyclopent-2-en-1-one (compound 64

Table 3 - Conditions of the Stability Assays

Human Plasma Solution	GSH Solution
CP (5µmol)	CP (5µmol) + GSH (2equiv.)
Standard Solution (1.3 equiv.)	Standard Solution (1.3 equiv.)
Solvent (6% Plasma + 14% Phosphate buffer + 80% DMSO)	Solvent (20% GSH solution in Phosphate buffer + 80% DMSO)

6

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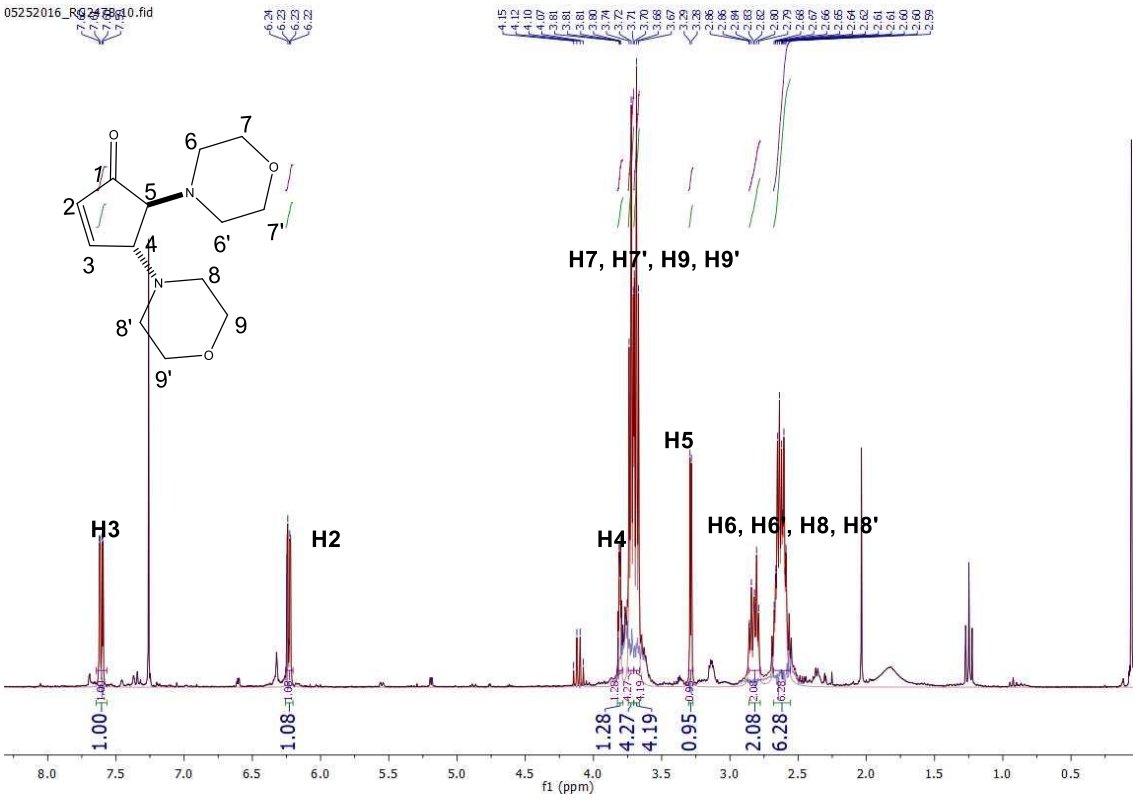
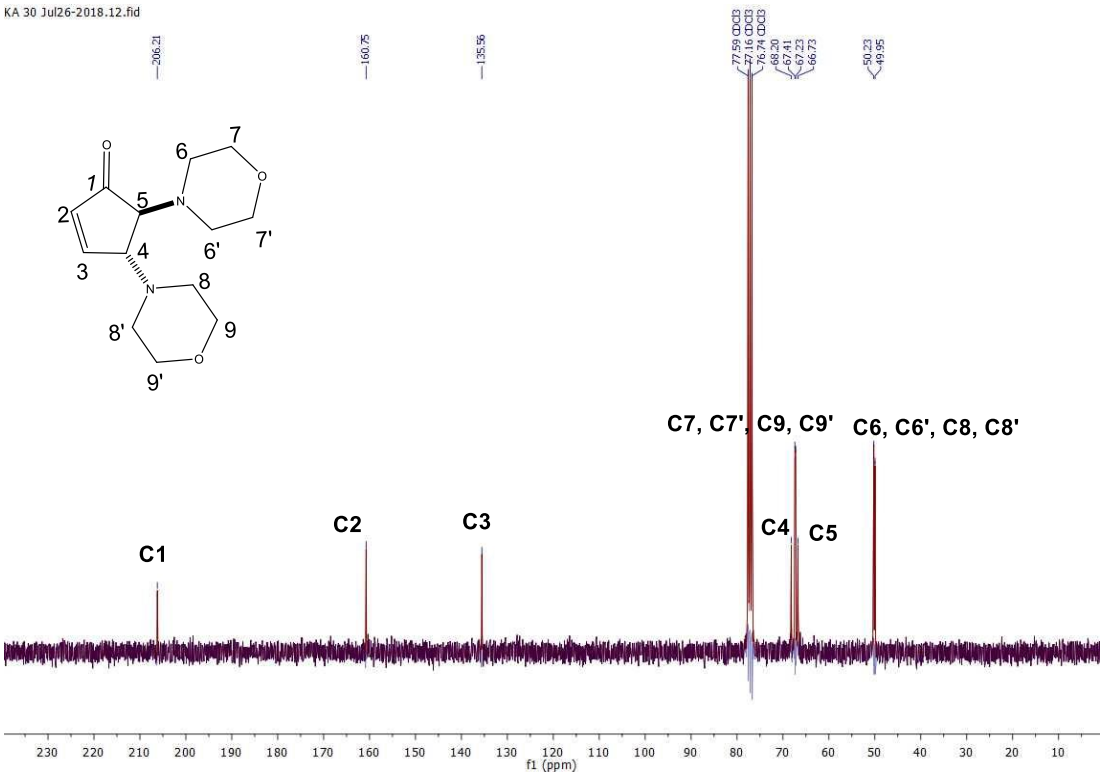
7

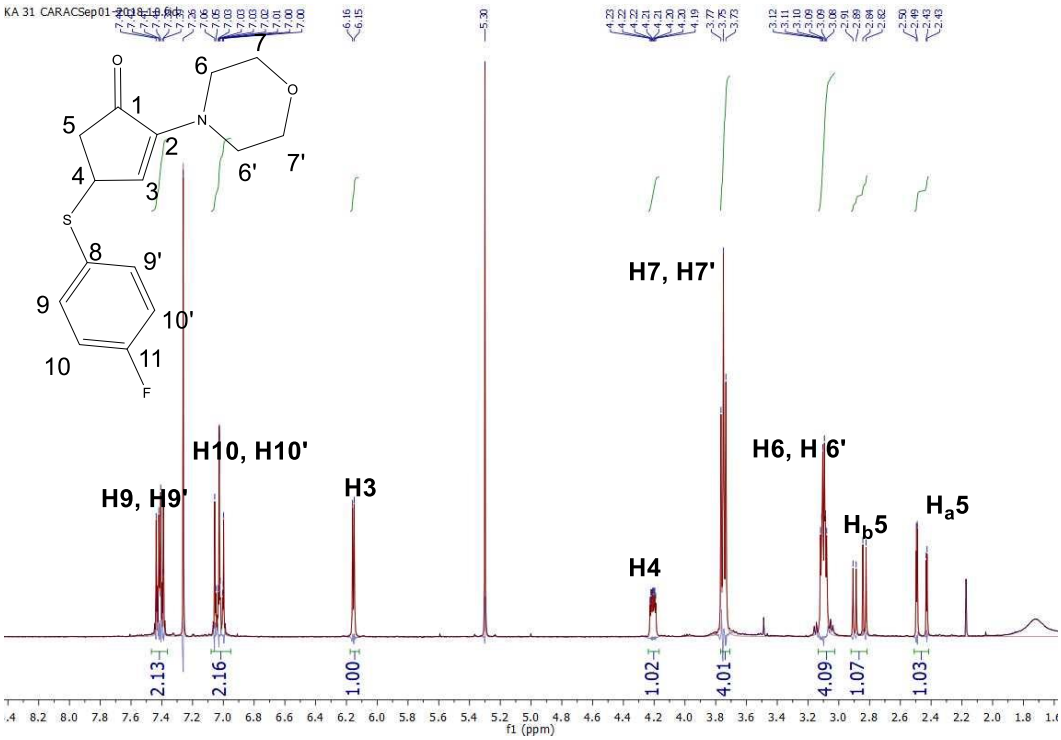
Annexes



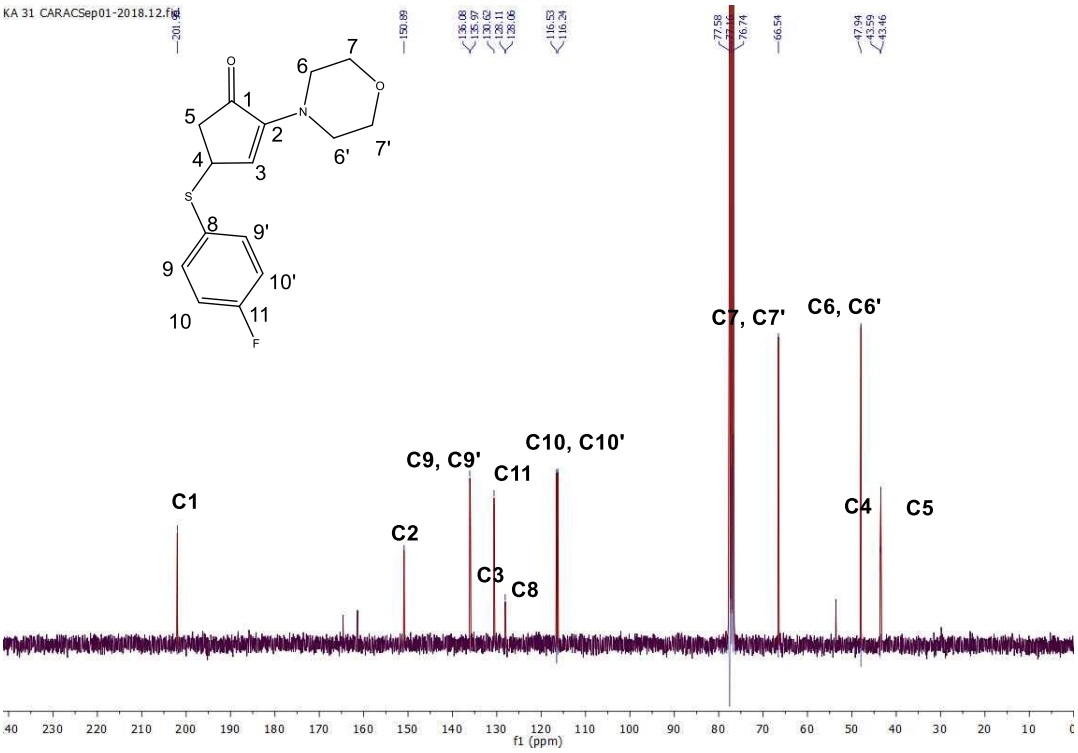
Appendix 7.1 - ^{1}H NMR spectra at 300MHz in $CDCl_3$ of *trans*-4,5-dimorpholinocyclopent-2-en-1-one



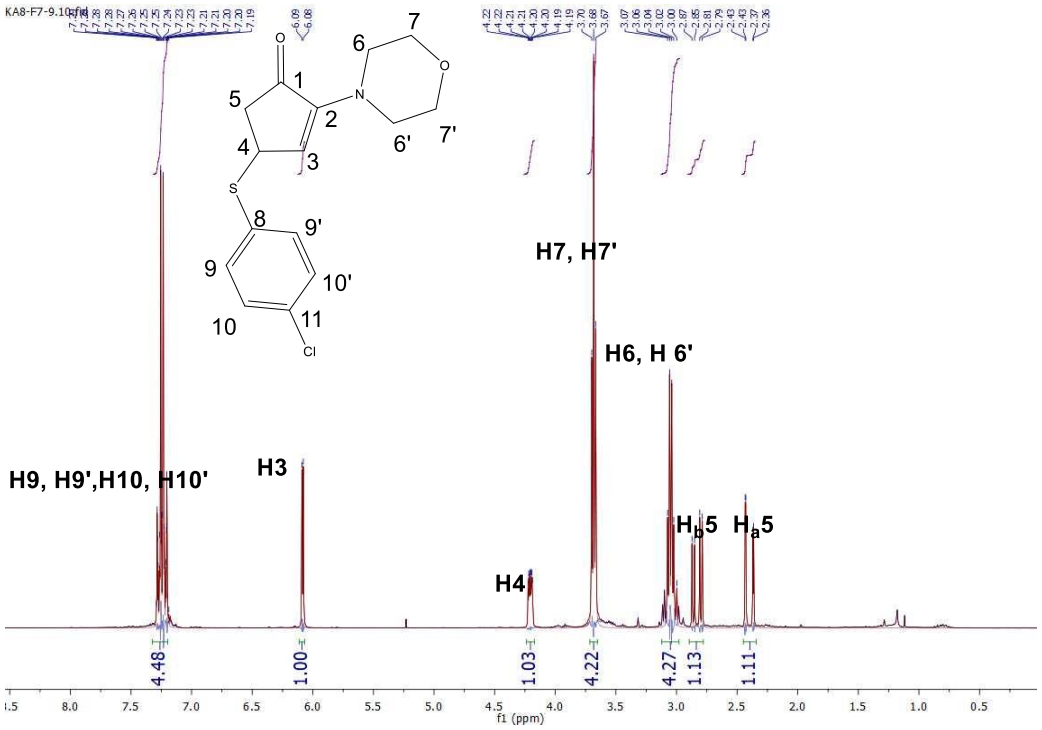
Appendix 7.2 - ^{13}C NMR spectra at 100MHz in $CDCl_3$ of *trans*-4,5-dimorpholinocyclopent-2-en-1-one



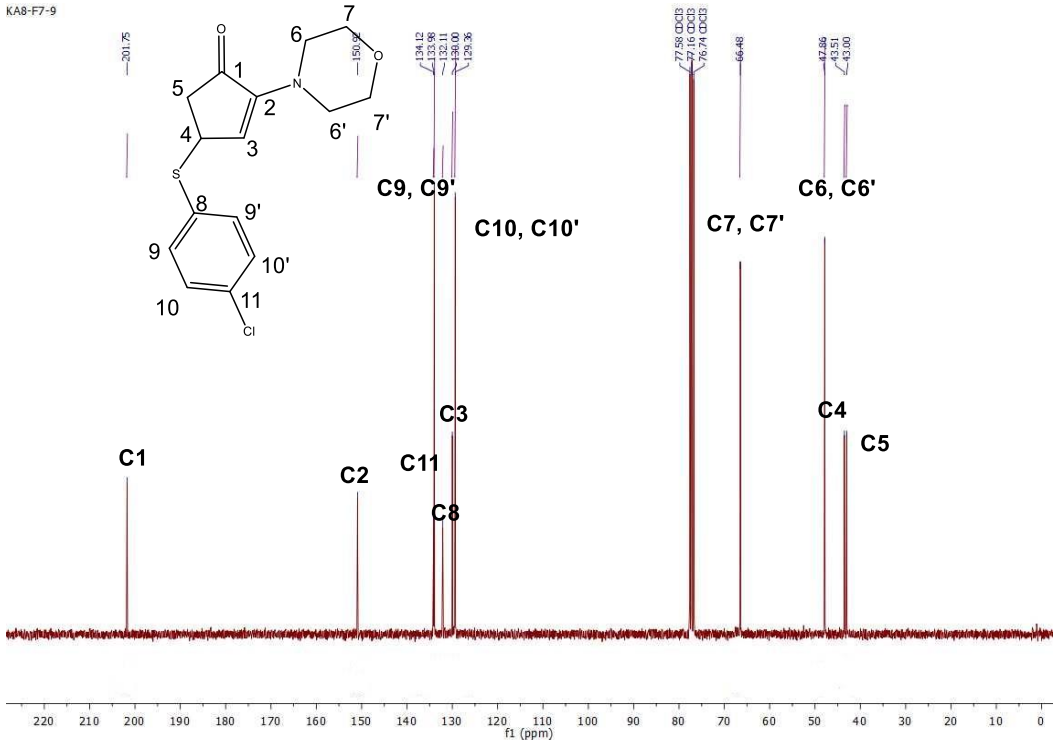
Appendix 7.3 - ^{1}H NMR spectra at 300MHz in $CDCl_3$ of 4-((4-fluorophenyl)thio)-2-morpholinocyclopent-2-en-1-one



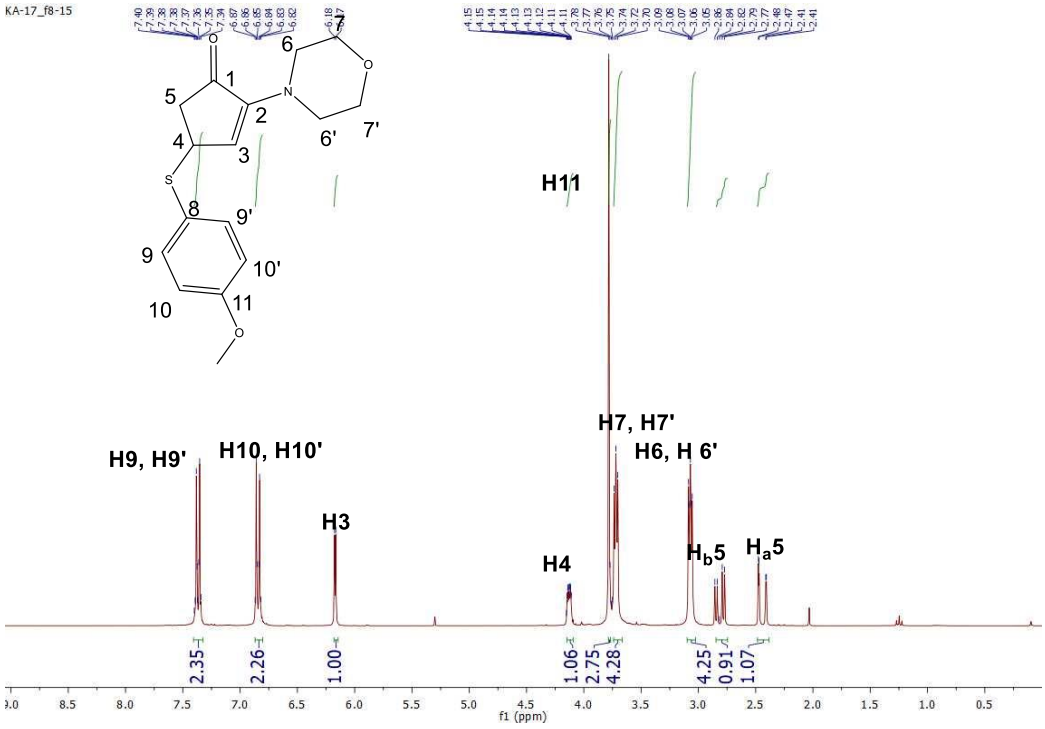
Appendix 7.4 - ^{13}C NMR spectra at 100MHz in $CDCl_3$ of 4-((4-fluorophenyl)thio)-2-morpholinocyclopent-2-en-1-one



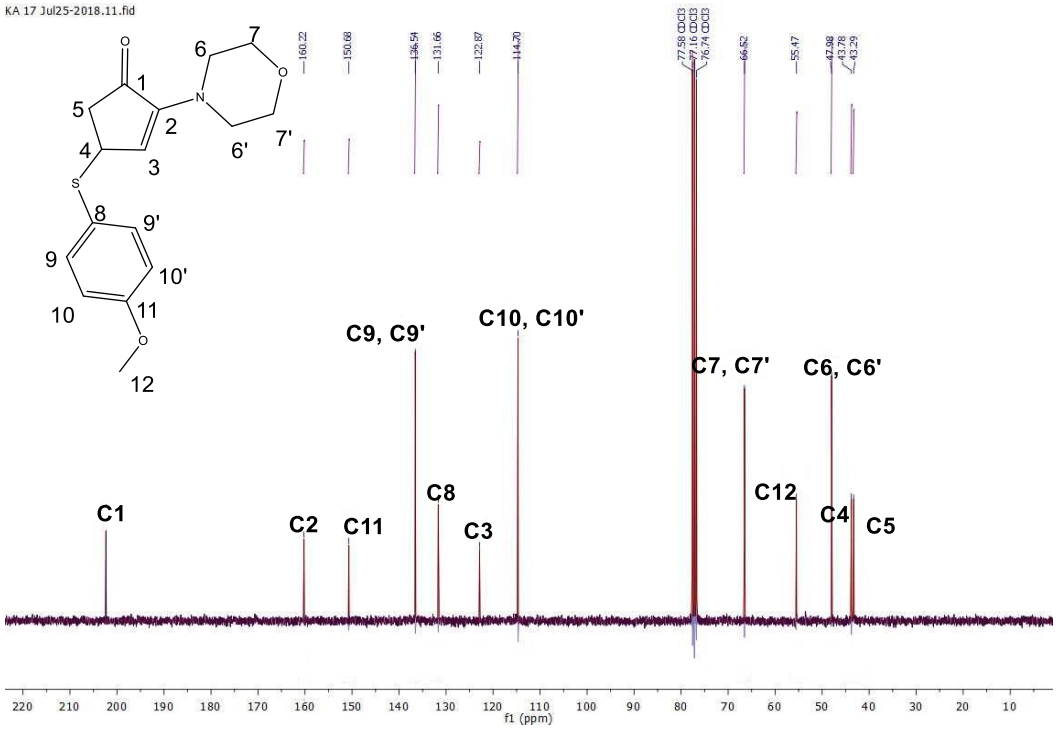
Appendix 7.5 - ^{1}H NMR spectra at 300MHz in $CDCl_3$ of 4-((4-chlorophenyl)thio)-2-morpholinocyclopent-2-en-1-one



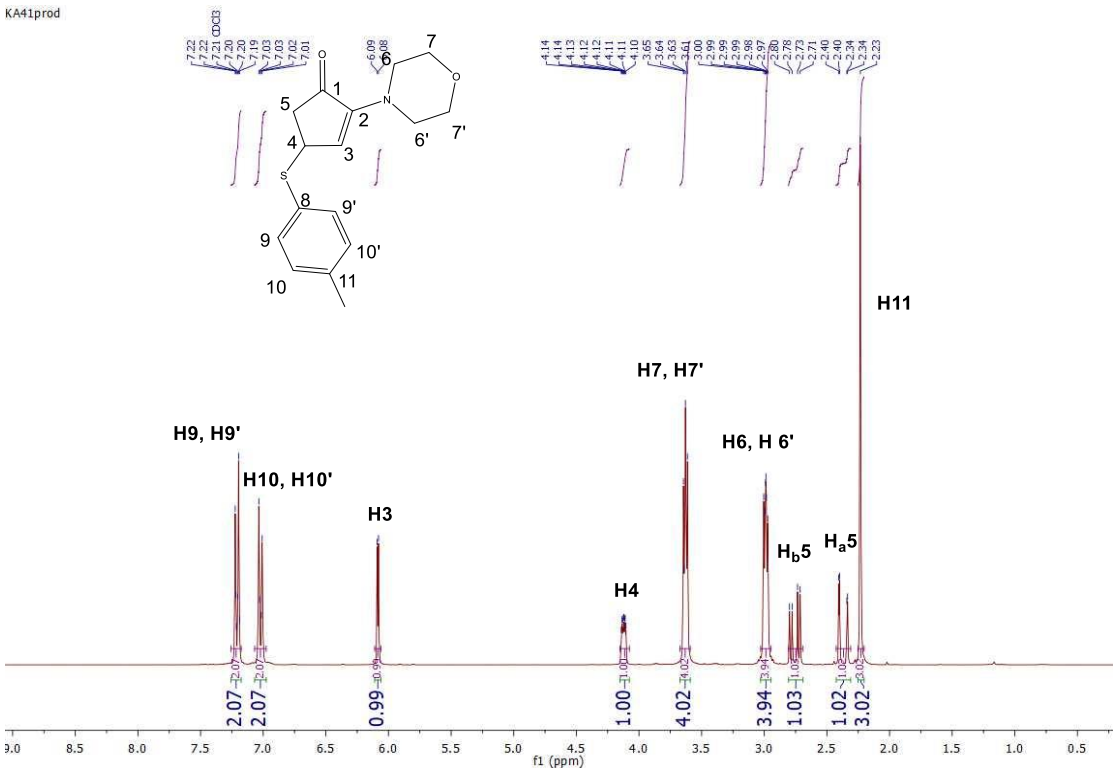
Appendix 7.6 - ^{13}C NMR spectra at 100MHz in $CDCl_3$ of 4-((4-chlorophenyl)thio)-2-morpholinocyclopent-2-en-1-one



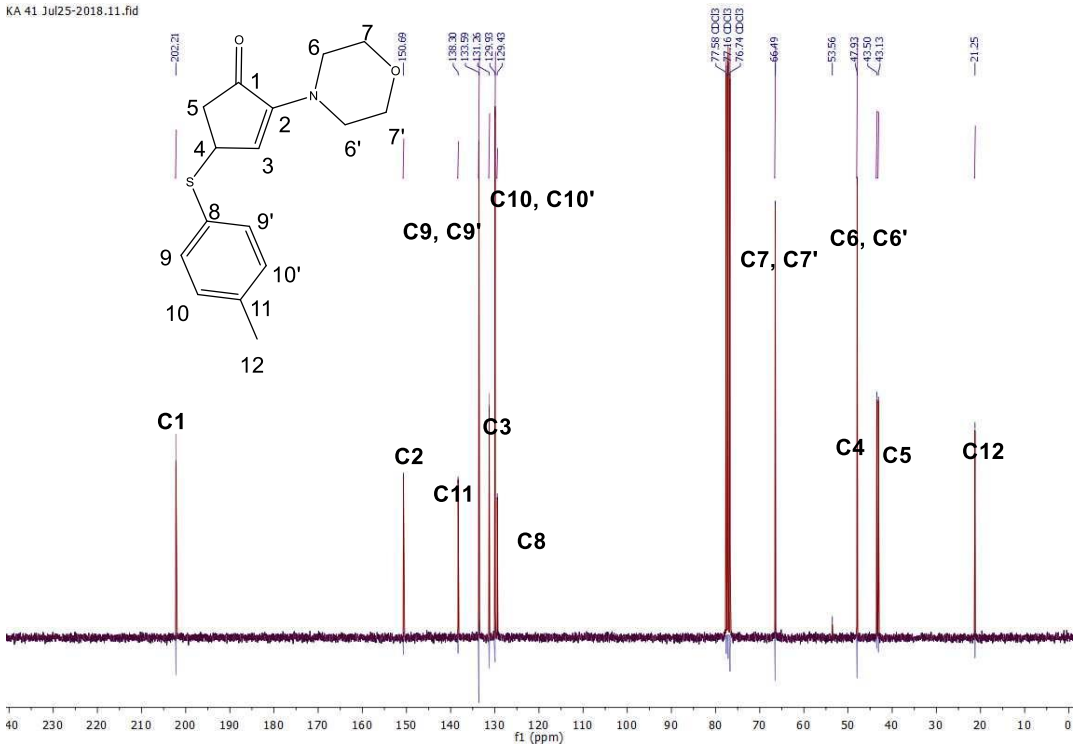
Appendix 7.7 - ^{1}H NMR spectra at 300MHz in $CDCl_3$ of 4-((4-methoxyphenyl)thio)-2-morpholinocyclopent-2-en-1-one



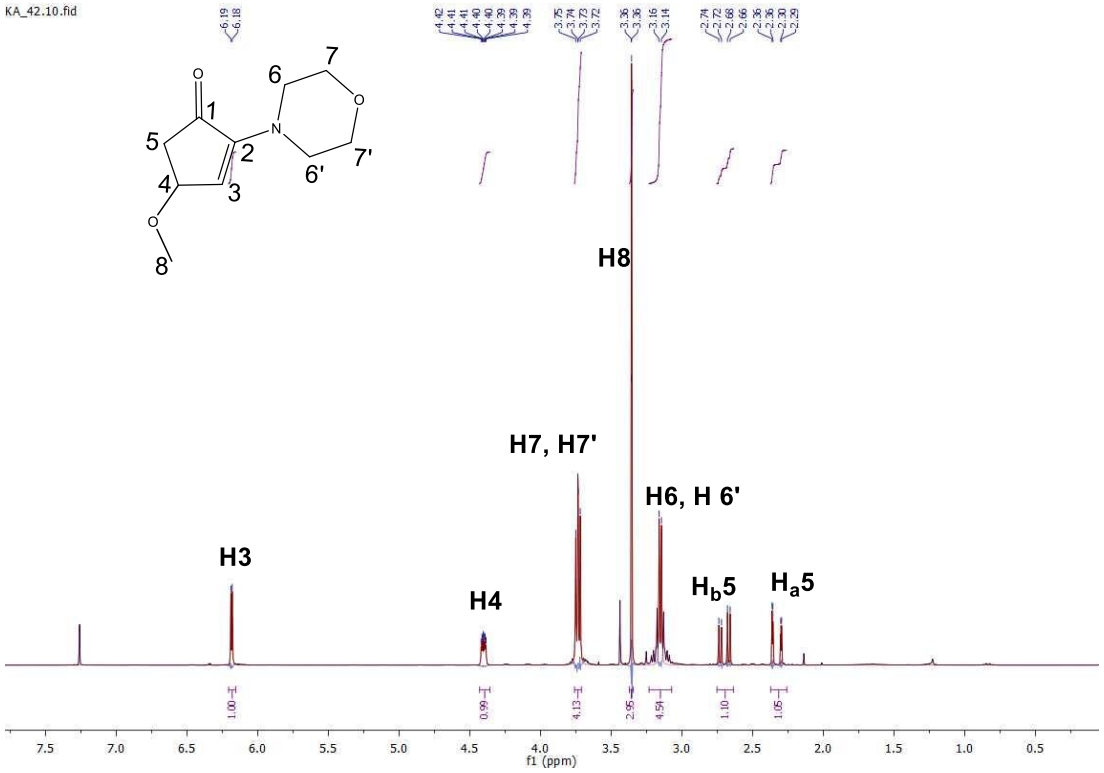
Appendix 7.8 - ^{13}C NMR spectra at 100MHz in $CDCl_3$ of 4-((4-methoxyphenyl)thio)-2-morpholinocyclopent-2-en-1-one



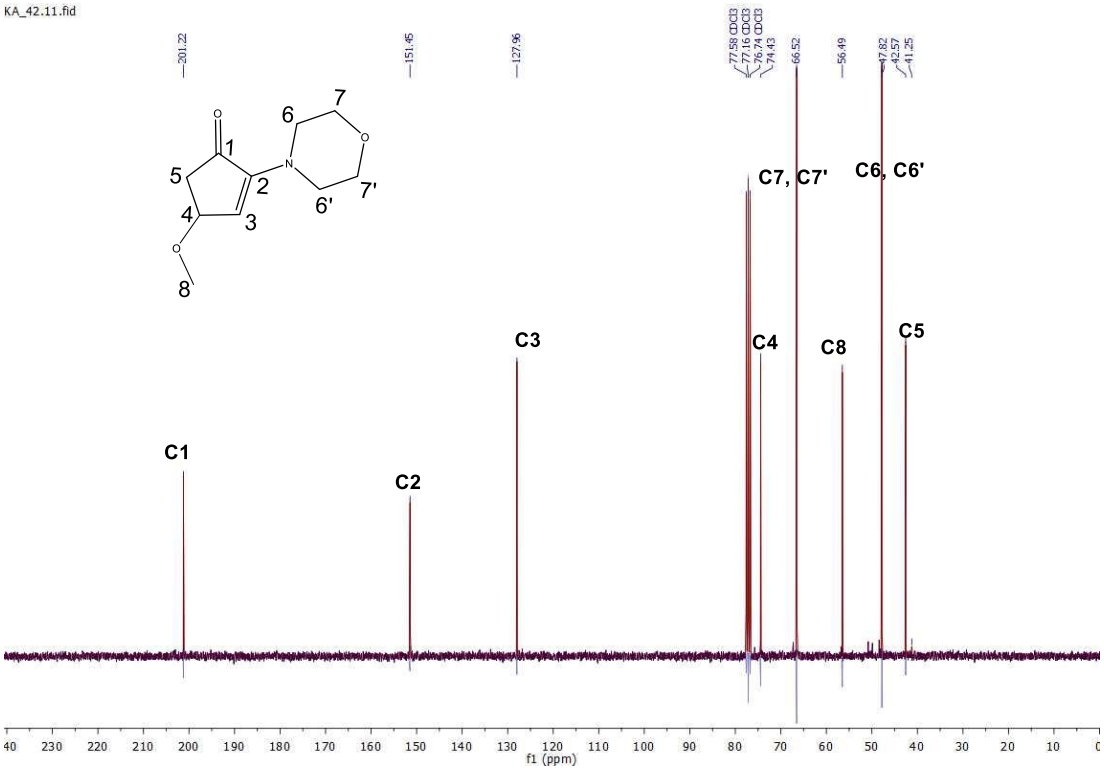
Appendix 7.9 - ^{1}H NMR spectra at 300MHz in $CDCl_3$ of 2-morpholino-4-(p-tolylthio)cyclopent-2-en-1-one



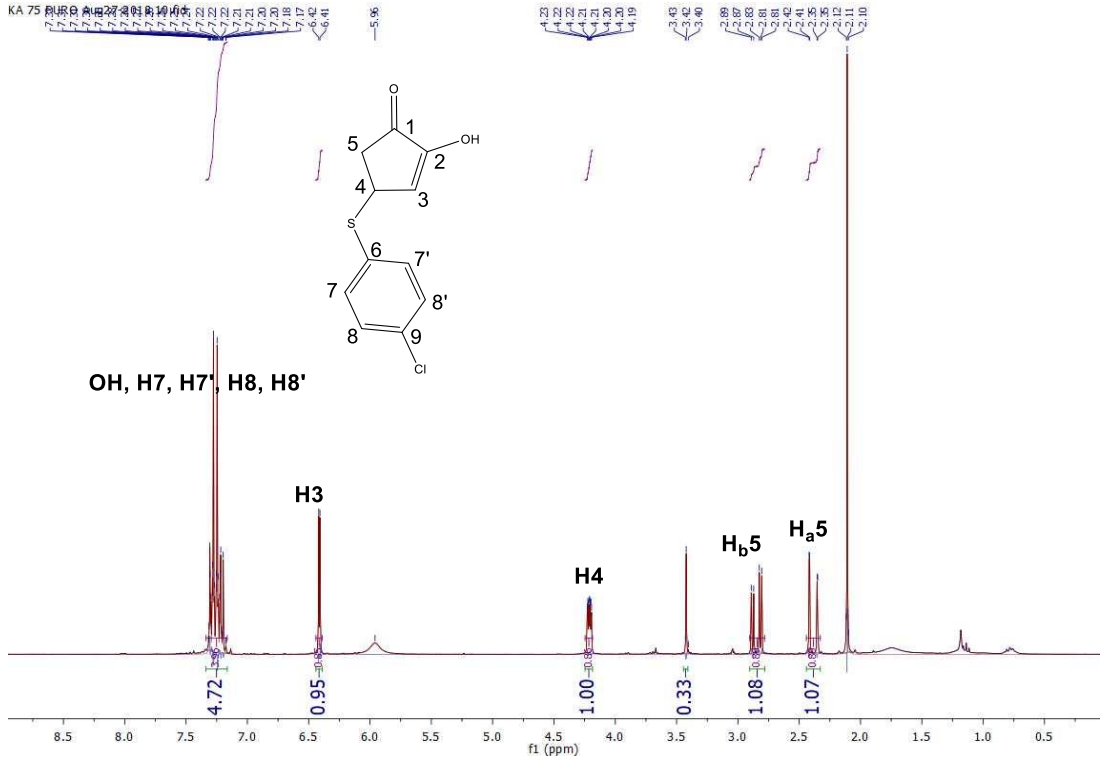
Appendix 7.10 - ^{13}C NMR spectra at 100MHz in $CDCl_3$ of 2-morpholino-4-(p-tolylthio)cyclopent-2-en-1-one



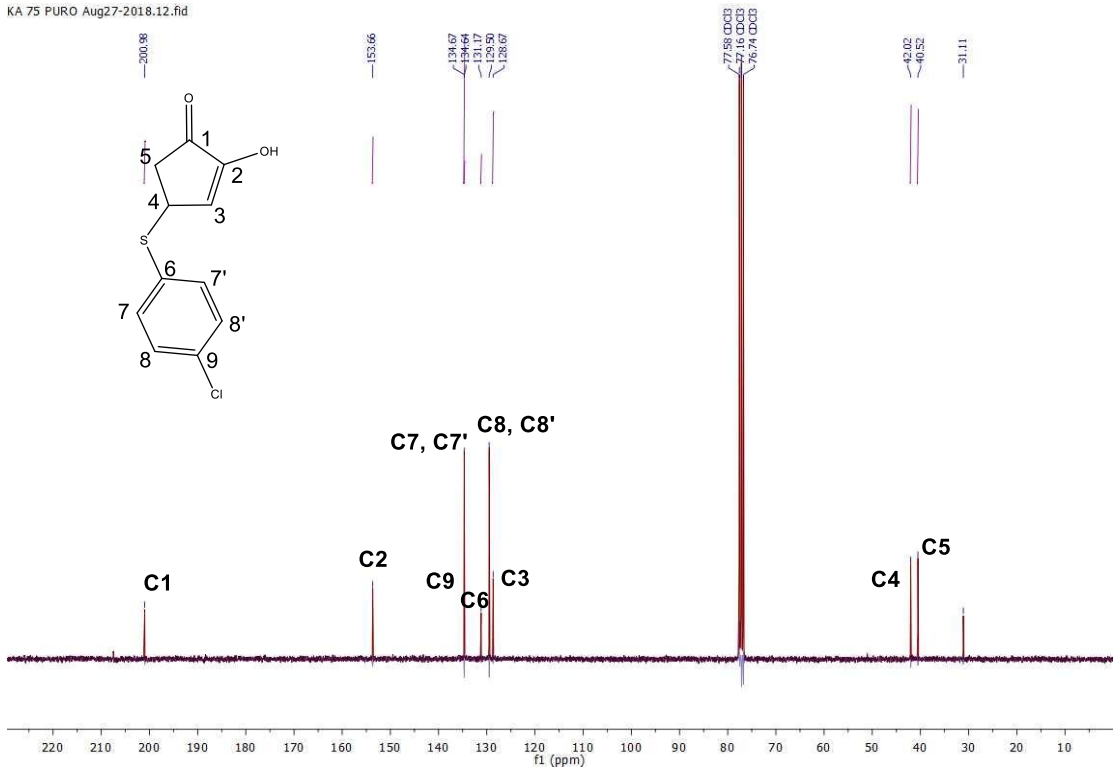
Appendix 7.11 - ^{1}H NMR spectra at 300MHz in $CDCl_3$ of 4-methoxy-2-morpholinocyclopent-2-en-1-one



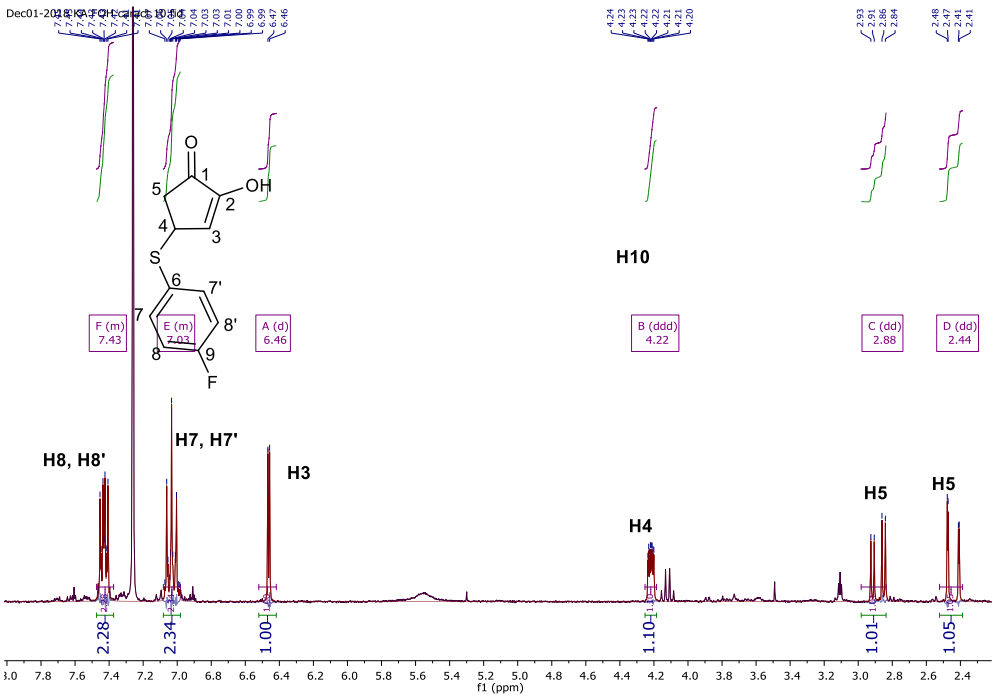
Appendix 7.12 - ^{13}C NMR spectra at 100MHz in $CDCl_3$ of 4-methoxy-2-morpholinocyclopent-2-en-1-one



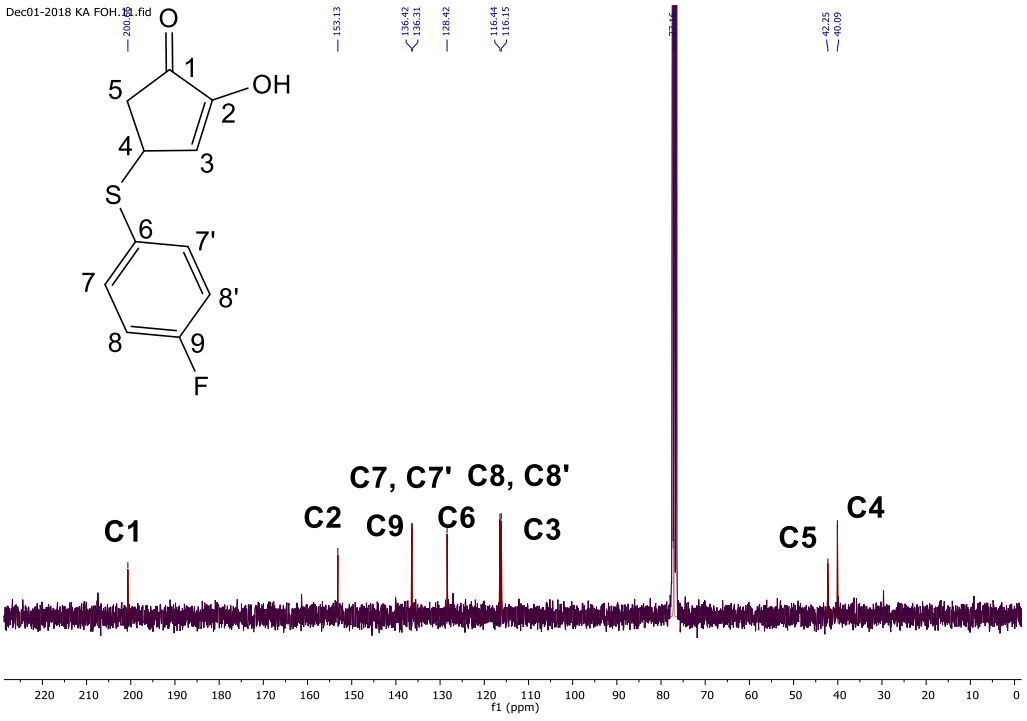
Appendix 7.13 - ^{1}H NMR spectra at 300MHz in $CDCl_3$ of 4-((4-hlorophenyl)thio)-2-hydroxycyclopent-2-en-1-one



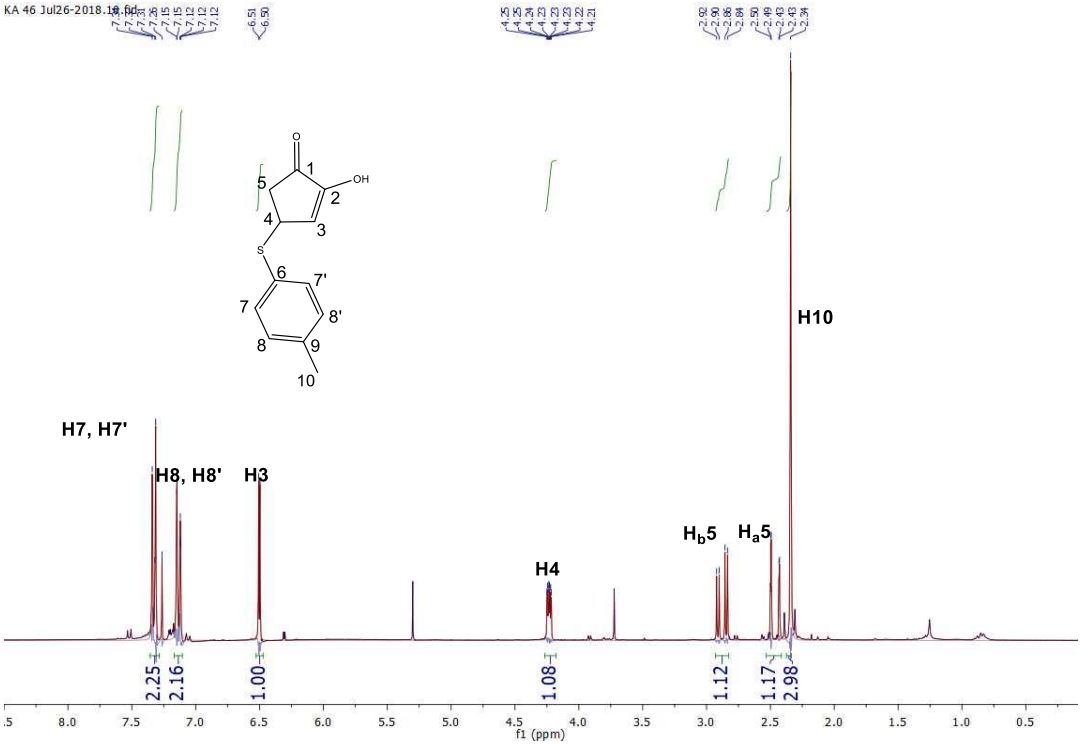
Appendix 7.14 - ^{13}C NMR spectra at 100MHz in $CDCl_3$ of 4-((4-hlorophenyl)thio)-2-hydroxycyclopent-2-en-1-one



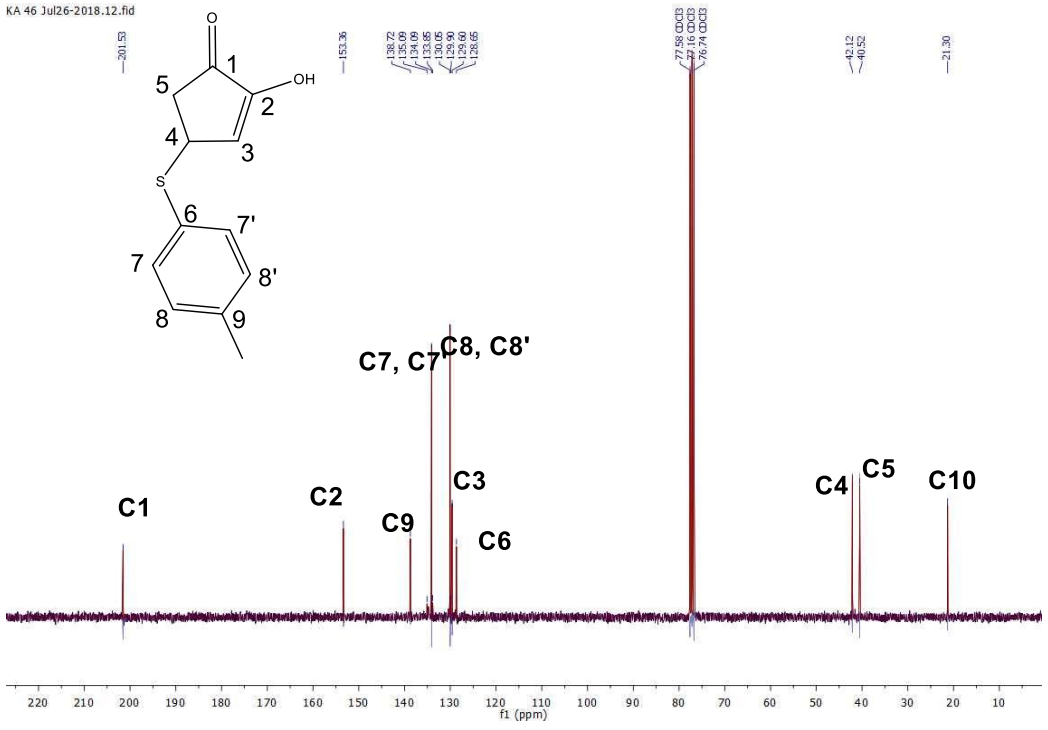
Appendix 7.15 - ^{1}H NMR spectra at 300MHz in $CDCl_3$ of 4-((4-fluorophenyl)thio)-2-hydroxycyclopent-2-en-1-one



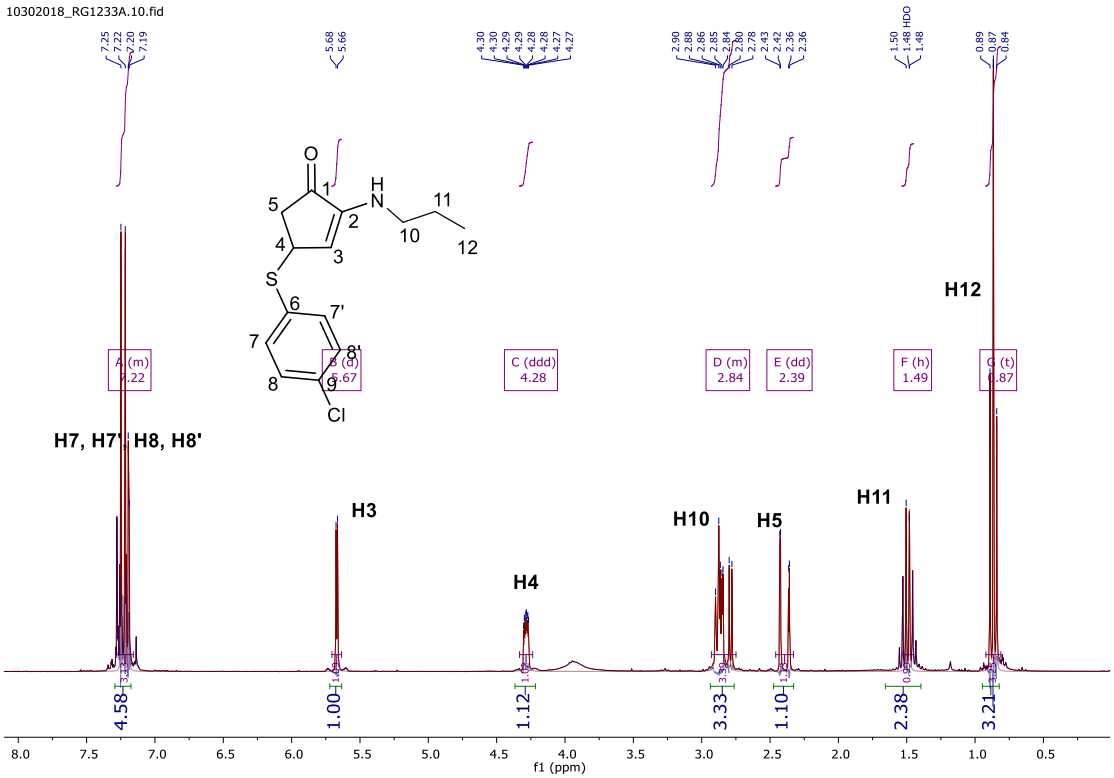
Appendix 7.16 - ^{13}C NMR spectra at 100MHz in $CDCl_3$ of 4-((4-fluorophenyl)thio)-2-hydroxycyclopent-2-en-1-



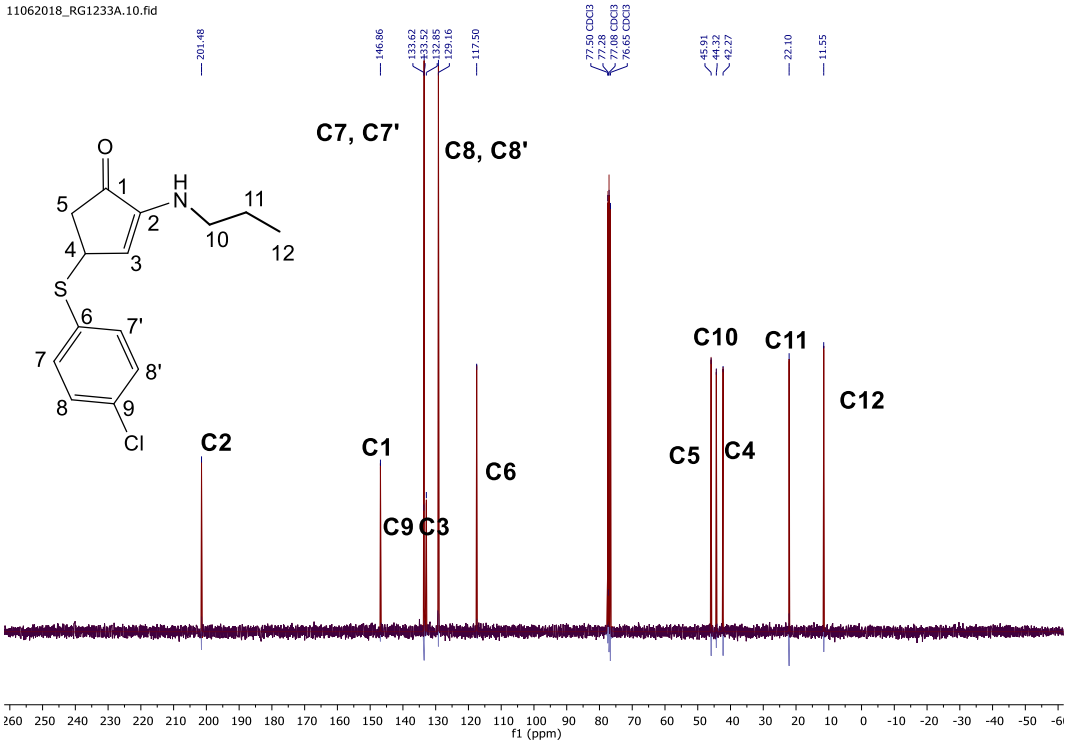
Appendix 7.17 - ^{1}H NMR spectra at 300MHz in $CDCl_3$ of 2-hydroxy-4-(p-tolylthio)cyclopent-2-en-1-one



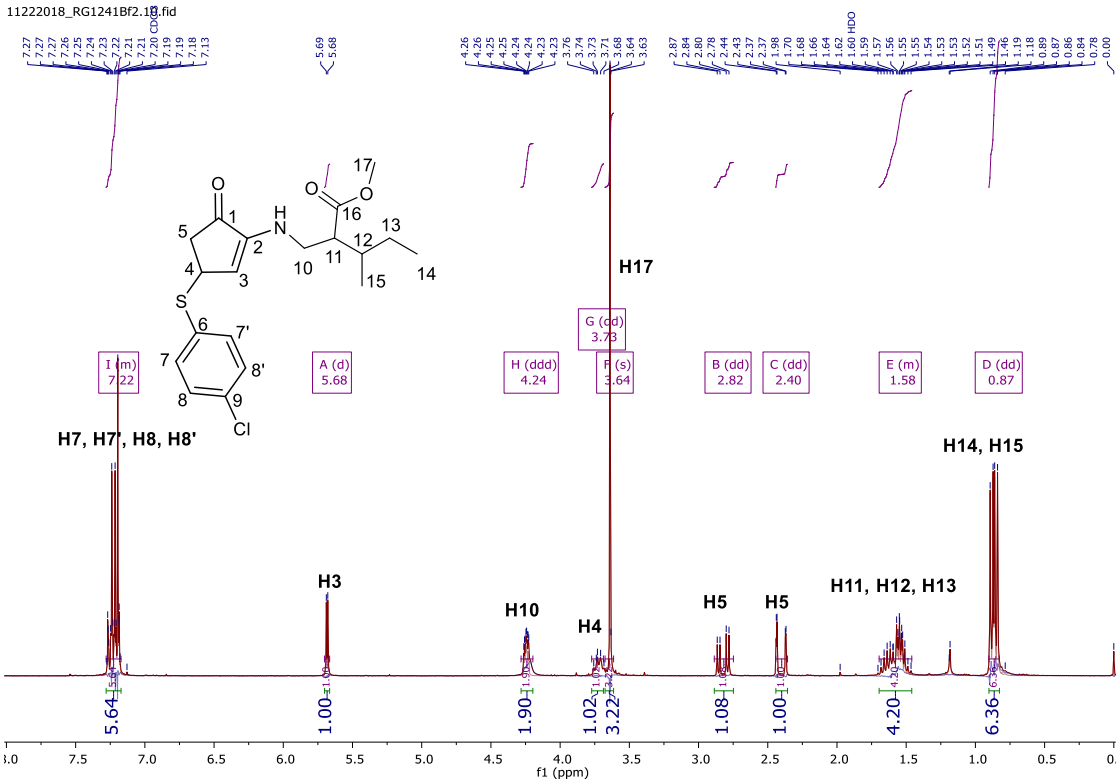
Appendix 7.18 - ^{13}C NMR spectra at 100MHz in $CDCl_3$ of 2-hydroxy-4-(p-tolylthio)cyclopent-2-en-1-one



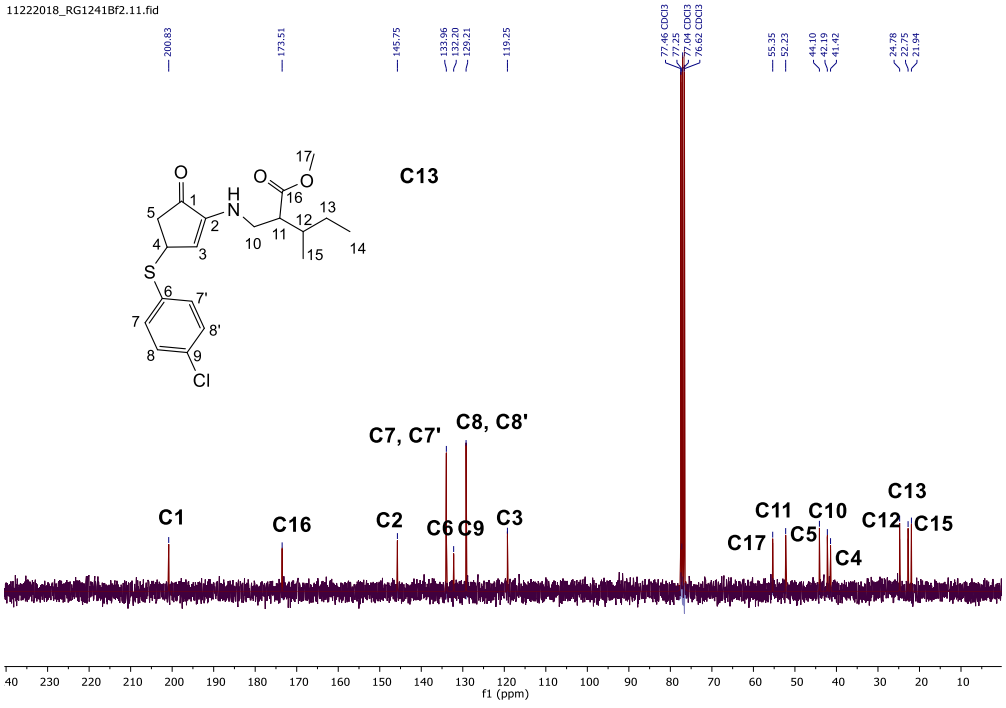
Appendix 7.20 - ^{1}H NMR spectra at 300MHz in $CDCl_3$ of 4-((4-chlorophenyl)thio)-2-(propylamino)cyclopent-2-en-1-one



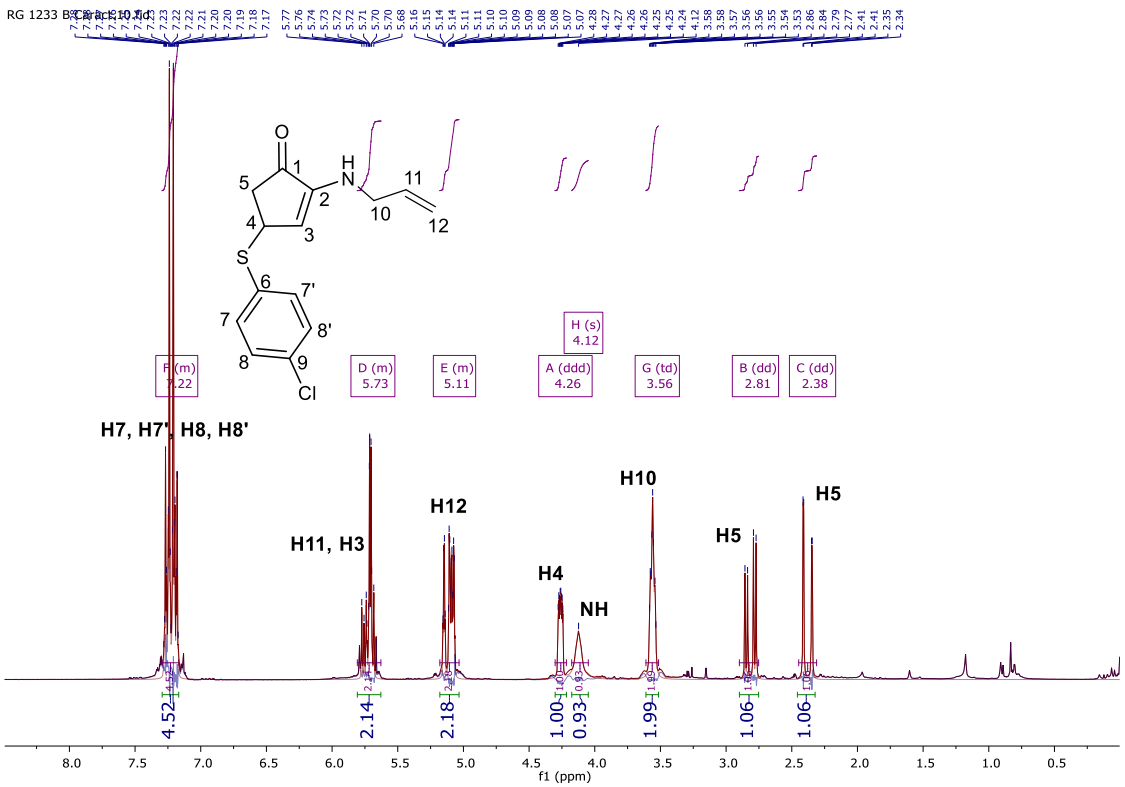
Appendix 7.21 - ^{13}C NMR spectra at 100MHz in $CDCl_3$ of 4-((4-chlorophenyl)thio)-2-(propylamino)cyclopent-2-en-1-one



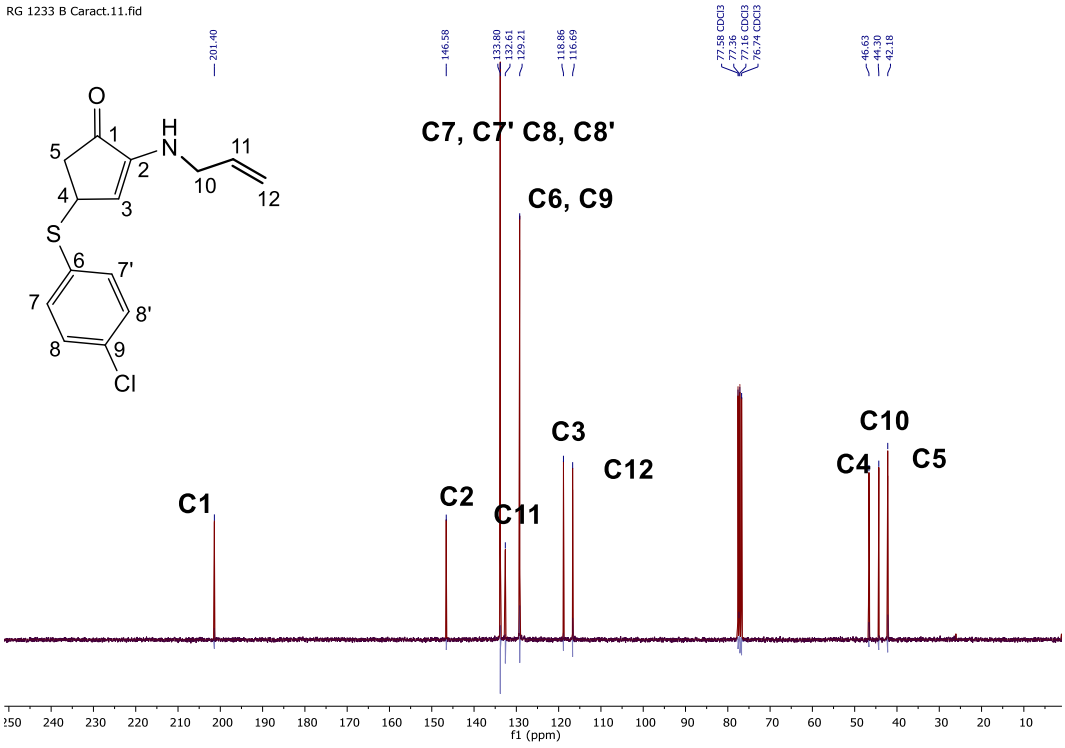
Appendix 7.22 - ^{1}H NMR spectra at 300MHz in $CDCl_3$ of 4-((4-chlorophenyl)thio)-2-(pentylamino)cyclopent-2-en-1-one



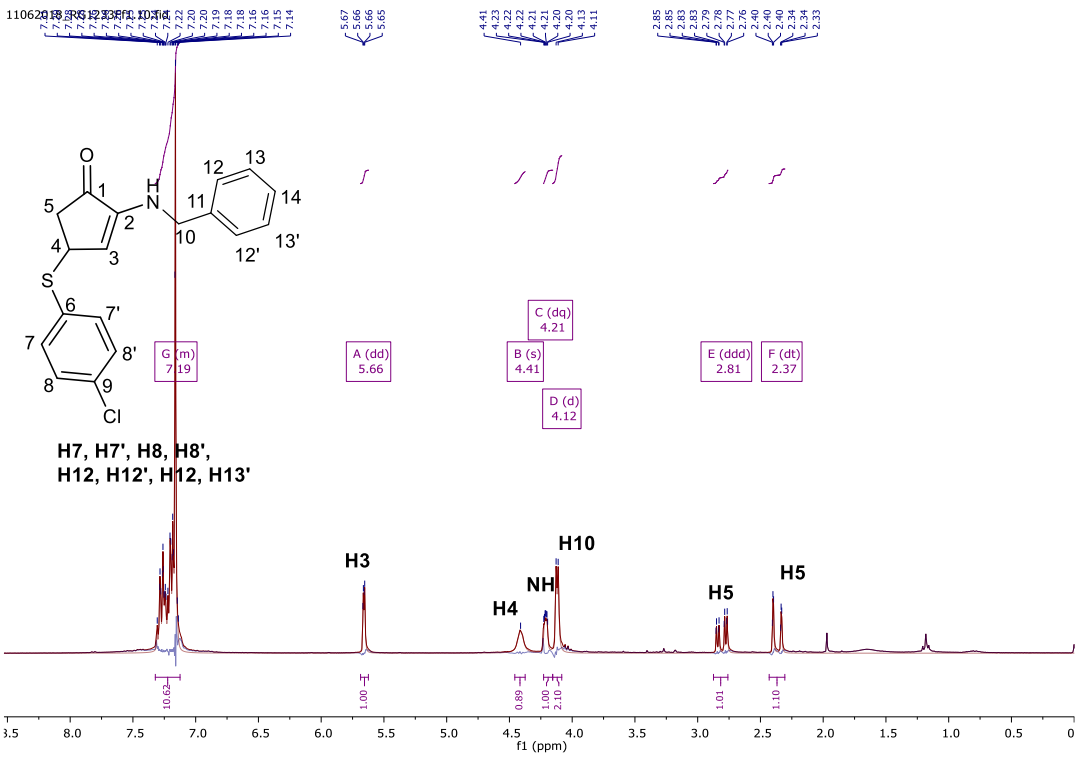
Appendix 7.23 - ^{13}C NMR spectra at 100MHz in $CDCl_3$ of 4-((4-chlorophenyl)thio)-2-(pentylamino)cyclopent-2-en-1-one



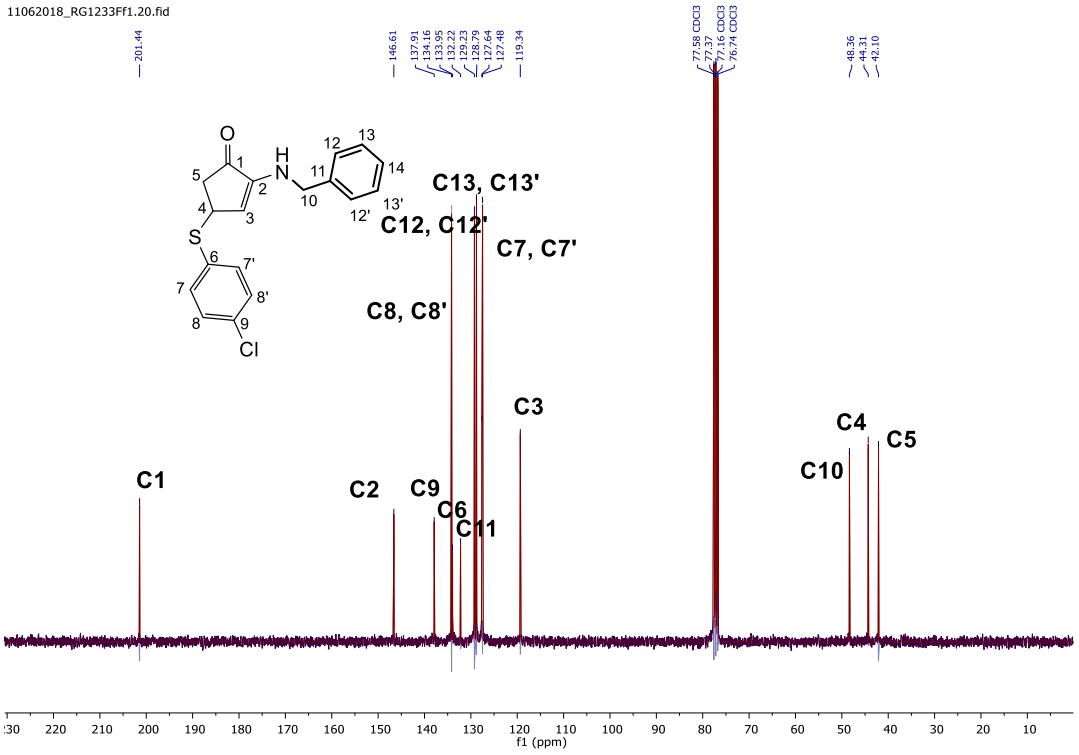
Appendix 7.24 - ^{1}H NMR spectra at 300MHz in $CDCl_3$ of 2-(allylamino)-4-((4-chlorophenyl)thio)cyclopent-2-en-1-one



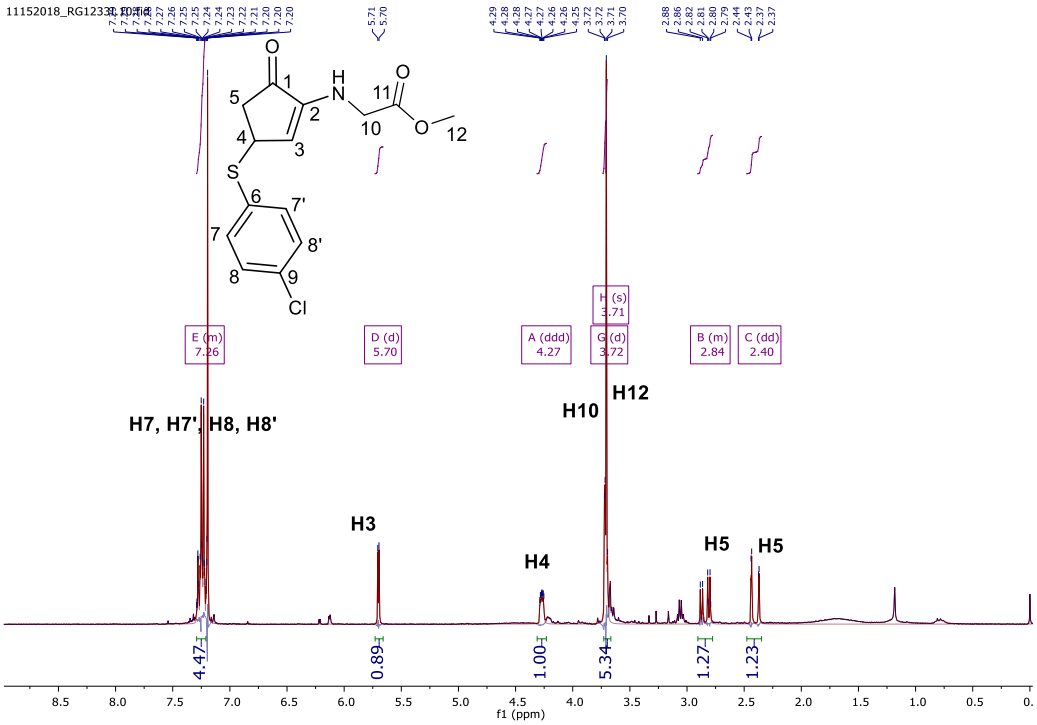
Appendix 7.25 - ^{13}C NMR spectra at 100MHz in $CDCl_3$ of 2-(allylamino)-4-((4-chlorophenyl)thio)cyclopent-2-en-1-one



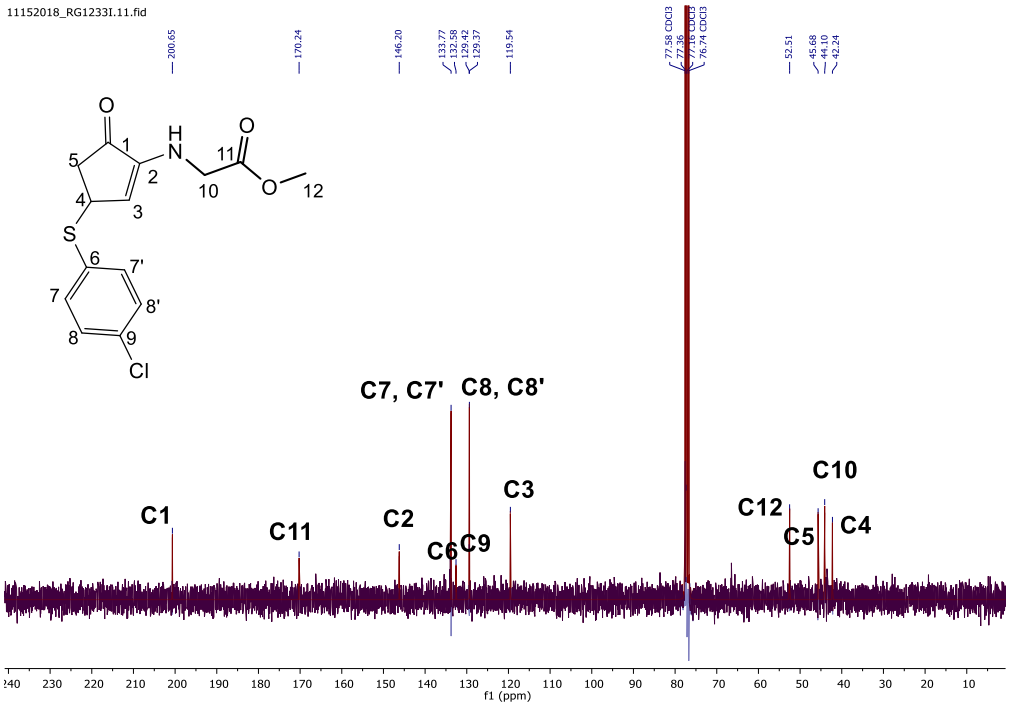
Appendix 7.26 - ^{1}H NMR spectra at 300MHz in $CDCl_3$ of 2-(benzylamino)-4-((4-chlorophenyl)thio)cyclopent-2-en-1-one



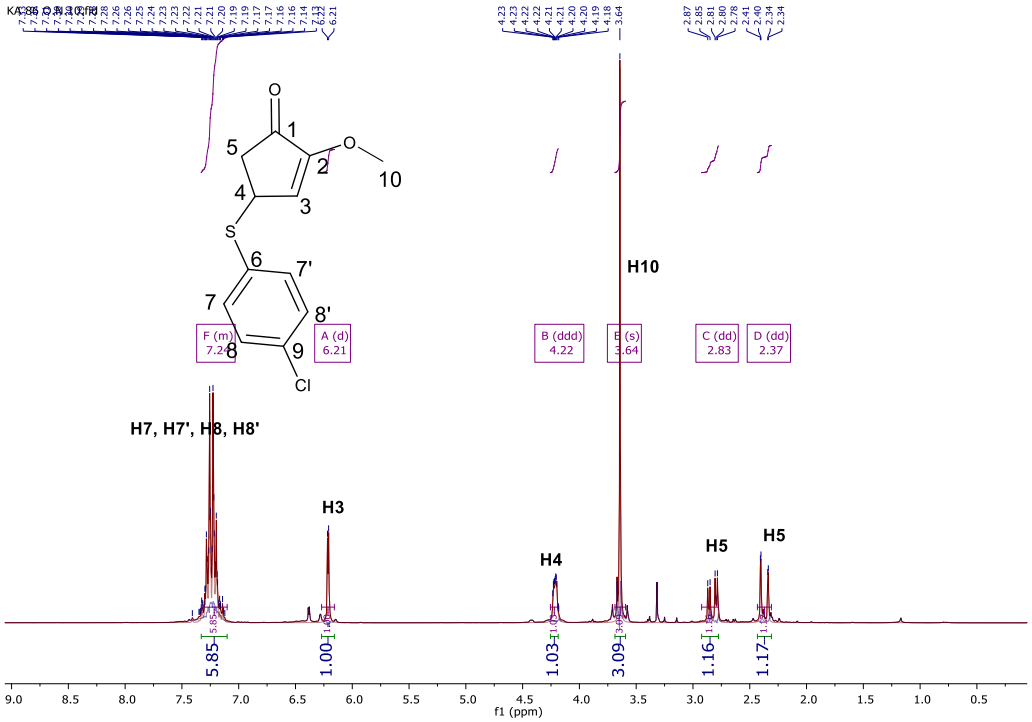
Appendix 7.27 - ^{13}C NMR spectra at 100MHz in $CDCl_3$ of 2-(benzylamino)-4-((4-chlorophenyl)thio)cyclopent-2-en-1-one



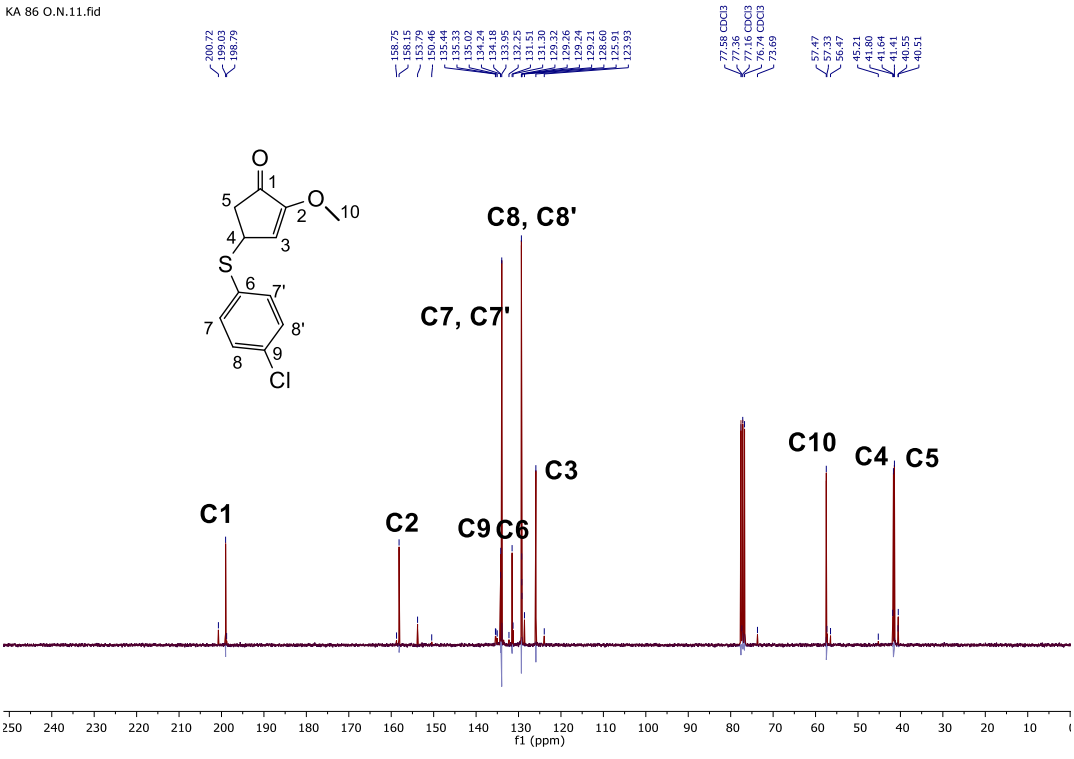
Appendix 7.28 - ^{1}H NMR spectra at 300MHz in $CDCl_3$ of methyl (3-((4-chlorophenyl)thio)-5-oxocyclopent-1-en-1-yl)glycinate



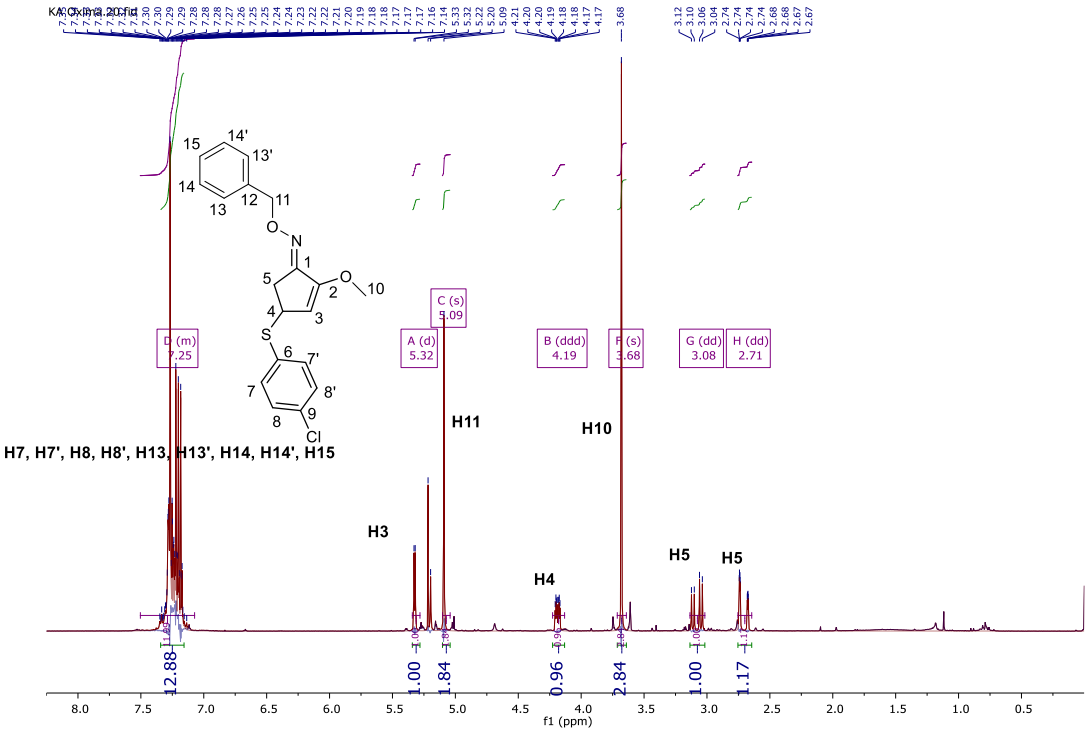
Appendix 7.29 - ^{13}C NMR spectra at 100MHz in $CDCl_3$ of methyl (3-((4-chlorophenyl)thio)-5-oxocyclopent-1-en-1-yl)glycinat



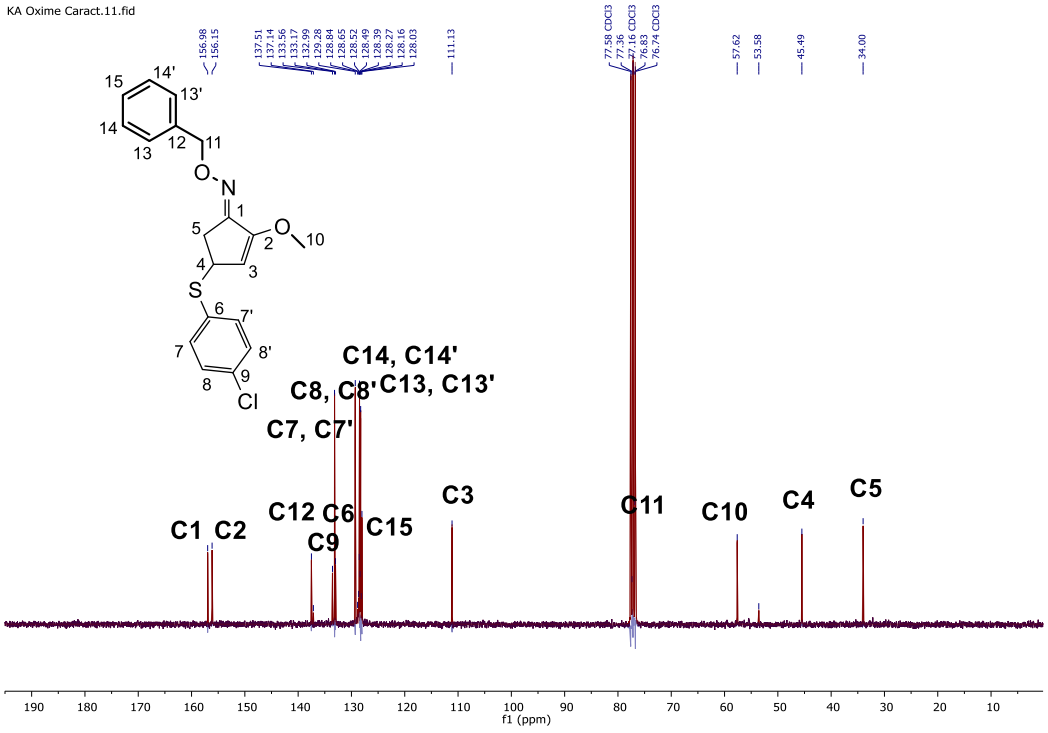
Appendix 7.30 - ^{1}H NMR spectra at 300MHz in $CDCl_3$ of 4-((4-chlorophenyl)thio)-2-methoxycyclopent-2-en-1-one's



Appendix 7.31 - ^{13}C NMR spectra at 100MHz in $CDCl_3$ of 4-((4-chlorophenyl)thio)-2-methoxycyclopent-2-en-1-one's



Appendix 7.32 - ^{1}H NMR spectra at 300MHz in $CDCl_3$ of E)-4-((4-chlorophenyl)thio)-2-methoxycyclopent-2-en-1-one O-benzyl oxime



Appendix 7.33 - ^{13}C NMR spectra at 100MHz in $CDCl_3$ of E)-4-((4-chlorophenyl)thio)-2-methoxycyclopent-2-en-1-one O-benzyl oxime